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Molecular Biological Characterization of Air Samples: A Survey of Four Strategically Important Regions

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14. ABSTRACT

Defense of overseas installations and personnel is a high priority for DoD and other U.S. Government agencies. In support of this requirement, the Joint Program Office for Biological Defense initiated an aggressive program incorporating the development of air-sampling and agent detecting devices, coined the Portal Shield/Port Biological Detection System. Since initiation of development, several iterations of the device have been developed and manufactured. However, in all iterations, operation remains essentially unchanged. Air is collected and any particulate matter interrogated for size and potential biological origin. The concentrated material is deposited in a liquid media and evaluated further for specific identification of agents. Field testing of Portal Shield modifications has progressed to operational evaluation and use overseas. Two United States airbases in Korea and two bases in southwest Asia were selected for these tests. In addition to conducting field testing of the equipment, it was decided to also collect ambient air samples in order to obtain ambient, normal background determinations in these strategically important regions over an extended period.

This report describes the analysis of samples collected over nearly 2 years, a total of roughly 2,100 individual samples. Sequence data are archived on a CD.

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EXECUTIVE SUMMARY

Defense of overseas installations and personnel is a high priority by DoD and other U.S. Government agencies. In support of this requirement the Joint Program Office for Biological Defense initiated a program undertaken an aggressive program incorporating the development of air sampling and agent detecting devices, coined the Portal Shield/Port Biological Detection System. Since initiation of development, several iterations of the device has been developed and manufactured. However, in all iterations, operation remains essentially unchanged. Air is collected and any particulate matter interrogated for size and potential biological origin. The concentrated material is then deposited in a liquid media and evaluated further for specific identification of agents.

Field-testing of Portal Shield modifications has progressed to operational evaluation and use overseas. Two United States airbases in Korea and two bases in southwest Asia were selected for these tests. In addition to conducting field testing of the equipment, it was decided to also collect ambient air samples in order to obtain ambient, normal background determinations in these strategically important regions over an extended period.

The goal was to ascertain 'background' from two dimensions. The first is the identification of biological warfare pathogens that exist naturally in the environment. The second is the total number of organisms collected at any period thus giving a representation of endemic amount of any given genus or species. Many pathogens, such as *Bacillus anthracis, Yersinia pestis,* and *Francisella tularensis* occur naturally throughout the world. Many are often endemic in rural farming regions, and pose very little clinical significance due to low rates of human occurrence and therefore low likelihood of reinfection. However, operational deployment of U.S. troops could potentially expose relatively large numbers of people to these endemic pathogens.
Deployment of sensitive, long-term detection devices requires prior establishment of background levels in order to differentiate endemic presence of naturally occurring organisms from purposeful dissemination of agents. If detection devices are deployed to a region of the world in which there are actual or threatened hostilities, knowledge of the background level potential threat agents is critical for a determination of biological agent use and imposition of protective measures. Therefore, knowledge of endemic background levels of organisms will prevent the unnecessary implementation of restrictive protective measure.

Another important value of knowledge of background organisms is the affect of naturally occurring organisms on detection assays deployed to the region. Phylogenetically related organisms naturally present in the area could potentially have a serious interfering affect on any detection methods used. Without compensating for these possibilities, the rate of false or positive reactions could be significantly increased. To address this issue, samples were collected throughout the year in each of four sites. The samples were assayed specifically for the presence of specific pathogenic organisms as well as likely naturally occurring agents closely related to likely threat agents.

This report describes the analysis of samples collected over nearly two years, a total of roughly two thousand one hundred individual samples. Sequence data is archived on a CD, available from JPO-BD.

DISCLOSURES

The opinions and assertions contained herein are not to be construed as official or reflecting the views of the Department of Defense or other agencies of the U.S. government. The use of trademark or brand names is not intended to endorse their use or exclusion.

1.0 INTRODUCTION

1.1 Objectives of study

The object of this study was to develop a method of generically identifying microorganisms by comparing sequences of DNA conserved among all bacterial species. Furthermore, to address the lack of information required to form the microbiological baseline a database was developed to accurately differentiate between the natural, endemic presence of pathogenic organisms from the deliberate release of biological agents. This analysis system was subsequently used to conduct a survey of bioflora from air samples collected in four (4) strategically important parts of the world.

1.2 Background

The threat of deliberate release of biological agents in either a military scenario or a terrorist act on a civilian population has been growing in recent years. The reality of using biological agents was accentuated recently by anthrax ladened letters addressed to members of Congress and news media. Early detection of chemical and biological agents is a high priority of the U. S. government.

Although the United States no longer has an offensive biological warfare program, a number of government agencies are aggressively pursuing research aimed at force protection from potential exposure. For example, the Department of Defense has several programs aimed at developing vaccines and prophylactic treatments to counter bioagent infection. The DoD and other agencies are also developing methods such as rapid detection assays and early warning systems to detect the use of biological agents by hostile nations or terrorist groups.

Many of the biological agents employed in biowarfare are occur naturally in most parts of the world. Furthermore, increased globalization and world travel have contributed to emerging human health issues from exposure to microorganisms where they have not previously posed threats. Therefore, in order to plan for adequate medical protection of U.S. forces stationed abroad and to discriminate naturally occurring human pathogens from deliberate human release of biological agents, we have developed an initial database reflecting "background" microflora in military-relevant regions of the world.

1.2.1 Historical use of biological agents

Pathogenic biological agents have been employed in warfare for hundreds of years. The first documented use was to ward off invading Tartars by the defenders of the Genoese seaport of Caffa (1). During the siege, the corpses of Tartars, who died of plague while besieging the town, were catapulted beyond the city walls causing the disease to spread into the city. The ensuing epidemic contributed to the eventual fall of the city. Another, later example of military use of biological agents, occurred during the Pontiac Rebellion in New England where Colonel Bouquet (a British officer) provided smallpox infected blankets to unsuspecting Indians at Fort Pitt, Pennsylvania. A similar plan was undertaken in the late 18th century when Tunisians threw plague-infected clothing into La Calle that was held by Christians. Later examples occurred during the American civil war when Union troops, advancing south into Maryland and other border states, were cautioned not to drink or eat

anything provided by unknown civilians due to fears of poisoning. Despite the warnings, there were numerous cases of soldiers becoming ill after eating or drinking. Also, the Confederate troops late in the war (retreating in Mississippi) left dead animals in wells and ponds with the intention of rendering these water sources unusable to advancing Union troops. More insidious, was the attempt by Dr. Blackburn, a future governor of Kentucky, who attempted to infect clothing with smallpox and yellow fever and sell it to unwary Union troops. However, because of the normally widespread disease rate and mortality, the effectiveness of these attempts is difficult or impossible to accurately assess.

Employment of biological warfare in military strategy grew coincidentally with the increasing scope of warfare, especially after the Industrial Revolution. The advent of the germ theory permitted the elucidation and development of bacteriological methods that permitted refinement of biological agents, which were more effective at incapacitating opposing forces while minimizing the loss of friendly forces. In more recent times, it has been postulated, although not firmly documented, that the Germans used pathogenic bacteria to infect cattle and horses leaving U.S. ports bound for allied countries (2). However, it wasn't until the discovery of the apparent use of cholera, dysentery, typhoid, plague, anthrax and paratyphoid by the Japanese against Chinese troops that the U.S. decided to conduct research in biological warfare and establish a biological warfare capability. In 1941 the Secretary of War asked the National Academy of Sciences to appoint a committee, designated the Biological Warfare Committee. In 1944, the biological warfare program was transferred to the War Department. Initially, the army's biological warfare program was centered at Edgewood Arsenal in Edgewood, MD. In 1943, laboratories were established at Camp Detrick (now Fort Detrick) near Frederick, Maryland. The Vigo Ordnance Plant near Terre Haute, Indiana was converted into a biological agent and weapon production plant. Later, when the Vigo plant was closed after World War II, the Pine Bluff Arsenal was selected to be the new biological agent production site.

Early use of biological agents depended a great deal on the behavior of opposing forces. However, relying on soldiers to drink from a pond or well left too much to chance. Therefore, as additional countries developed biological agents, more work was put into the delivery mechanism. Methods were developed to deliver agents on a larger scale, with greater precision and with more predictable results. For example, exploding devices were created for dispersion of agents as clouds upwind of enemy forces. The soldiers would then become ill after exposure to the agents (3). As aerial delivery was perfected, the wind became the primary source of dissemination.

During the cold war, both sides could field mature ordinance delivery systems where chemical and biological agents could be relatively distributed over military or civilian population centers with concomitant high casualty rates. Furthermore, not only were delivery systems improved but agents were selected to be more compatible with newly created mass-delivery methods. During the U.S. offensive program period, despite many biological agents being standardized and delivery systems developed, only a few biological weapons were actually developed and standardized.

1.2.2 Focus of current efforts by the United States in standoff protection in biowarfare defense.

The United States formal rejection of biological use in warfare was documented in 1972 with the multilateral signing of the Biological Weapons Convention (BWC). The Convention, which had 103 signatory nations, including the United States and the former Soviet Union, prohibited the development, production, stockpiling or retention of biological agents and toxins. Exceptions were made for defensive purposes, such as vaccine development. Because of the Convention, the United States disestablished its chemical/biological arsenal, which had been stockpiled over the course of roughly twenty years but had never actually used.

Despite the 1972 Convention, a major concern still remains regarding verification and compliance with intentions enumerated by the BWC. In the late 1980's, defections from the Soviet Union permitted the United States intelligence community to claim that the Soviet Union had never disarmed and was still actively producing bioweapons in violation of the BWC (4). Further reports suggested that signatories to the convention were conducting genetic engineering studies on pathogenic bacteria, such as insertion of genes conferring multiple drug resistance, with the aim of producing organisms, which were more effective as bio-weapons. It was thought that signatories were developing "chimeras", between pathogens with the intention of combining the most deadly aspects of each chimeric partner to create a "super-plague."

The fall of the Soviet Union was coincident with the shutdown of the Soviet biological agent production facilities and other aspects of their war machine. Although the United States' greatest adversary was gone, a number of other hostile nations remained. Furthermore, many of these nations (i.e., Syria, Libya, Iran, Iraq and North Korea) are implicated in supporting or sympathizing with various non-government organizations (NGO). There is a concern that many of these nations would deploy and use biological weapons in an active land battle against the United States. For example, the United States, fearing that Iraq would deliver anthrax-containing scud missiles aimed at military forces or civilian populations during Desert Shield/Storm, expressly and pointedly warned Iraq not to use "Weapons of Mass Destruction" during the ground phase of Desert Storm in 1991.

International concern over use of weapons of mass destruction was heightened following the Aum Shinrikyo attack in the Tokyo subway on 20 March 1995 (5). During that incident twelve people died and fifty-five hundred people were injured and/or reported to hospitals with a variety of symptoms including nausea, perfuse sweating and inability to catch their breath. In addition, most were suffering from hysteria after learning they had been exposed to the nerve agent, sarin. Another case occurred in the Pacific Northwest in 1984, when the supporters of the Bhagwan Shree Rajneesh contaminated lettuce at a restaurant salad bar with *Salmonella typhi* (6). Roughly 750 patrons of the restaurant came down with typhoid fever, characterized by severe diarrhea. Fortunately, there were no deaths resulting from the bacterial contamination, however there were many reported illnesses requiring hospitalization. These incidents illustrate an important component to the use of these agents as terrorist weapons. It is not necessary to kill in order to be effective. Rather, the goal of a terrorist may merely be to incite terror by the threat of use.

Because the tools needed to make a biological weapon are simple and the technology needed is relatively straight forward, many fringe groups have contemplated or threatened their use. To make a bio-weapon, it is important for the weapons maker to be competent at working under aseptic conditions to prevent contaminating himself or contaminating his

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biological agents. These techniques are taught routinely in college introductory micro-biology courses. In order to completely protect biological agent workers, it would be desirable to be immunized against the agents being worked with, however, immunization is not essential as long as physical barriers, such as containment hoods, are used. Most pathogens can be isolated from nature with the correct screening and selection media. The knowledge and equipment to carry out microbial isolations are available from a university or even a high school microbiology laboratory course.

Because of the ease of obtaining and delivering many infectious organisms, U.S. overseas air bases, army bases, depots and ports are particularly vulnerable. Similarly vulnerable are U.S. civilians stationed overseas, as well as tourists. Terrorist groups or agents of a rouge nation may be able to deliver biological agents to soldiers and sailors when they are on base or in their living quarters. The land immediately surrounding a facility may also be infiltrated and a biological agent released upwind. For example, a biological agent could be aerosolized upwind of a population center and the agent allowed to drift with the wind into the unsuspecting population. Prior immunization of military personnel would reduce or eliminate any harmful health effects. However, immunization of civilians, including dependents of military personnel, civilian workers and tourists is not yet practical. Additionally, there is the logistical issue of decontamination, which is both costly and time consuming. A sensor platform capable of monitoring collected air samples has been suggested to detect and provide early warning of agent release. Such a platform would enable early warning of downwind personnel and allow them to assume the appropriate defensive posture (i.e., donning protective gear or even merely moving away from the contaminating cloud).

Monitoring air surrounding ports and military bases has been assigned to the Joint Program Office for Biological Defense (JPO-BD). Two collection devices have been employed worldwide, the Interim Biological Agent Detector (IBAD) and the The Portal Shield/Port Biological Detection System (Portal Shield). Both of these devices are designed to collect large volumes air into a liquid buffer for subsequent analysis for the presence of biological agents. The IBAD system, although simple in design and construction, is not capable of automatic, analytic operation.

Unlike IBADS, the Portal Shield is a more complex early warning system. The JPO-BD, through the Advanced Concept Technology Demonstration (ACTD), has a six-year mission to develop and demonstrate a functional early warning device. Portal Shield monitors atmospheric conditions, the biological load in the air and automatically analyzes samples for the presence of certain pathogens (7). The system contains a particle size counter, which assesses the number and relative size of particles in collected air samples. Bacteria, therefore, which are generally between 1-10 microns in size, are first discriminated based on size. Potential biological particles are further evaluated for the presence of tyrosine residues by absorption at 210 nm.

Alarm algorithms have been developed such that a dramatic increase in the level of biological particles above background diverts the airflow into a wetted-wall cyclone that captures the particles in buffer. The sample is then analyzed using an immunochromato-graphic assay system (specific assay for a particular pathogen or toxin). Alternatively, the sample can be archived, by automatically diverting the sample into a collection vial. The sampler has an onboard GPS, as well as, a meteorological station to monitor air temperature,

humidity, wind direction and atmospheric pressure. These parameters are recorded with time stamps and retained in a permanent database.

Many pathogenic organisms likely to be used in a biological agent delivery system are endemic in areas where U.S. forces could potentially be engaged. Many pathogens are normally found in soil or infected wild and/or domesticated animals. Many organisms, such as *Bacillus anthracis*, require a relatively high infective dose in order to produce disease in humans. Trace amounts of naturally occurring organisms, however, including *B. anthracis*, are easily detectable, in many parts of the world, using newly developed technology. The sensitive detection limits inherent in technologies, such as polymerase chain reaction (PCR), may present serious caveats in using data derived from them to determine whether a deliberate release of biological agents occurred. The presence of specific organisms may only represent natural, endemic organisms. Samples may contain organisms that are genetically or immunologically closely related to threat agents, leading to a false positive result. Conversely, the presence of closely related organisms may block or interfere with molecular or immunological detection methods, yielding false negative results.

2.0 TECHNOLOGICAL APPROACH

2.1 Collection Systems

The purpose of the sample collection was focused on determining the "background" microflora within a geographical region. To accomplish this, air samples were collected during the course of the year at each location. Air samples were collected, via the Portal Shield or Interim Biological Agent Detector (IBAD) into phosphate buffered saline (PBS) with sodium azide as preservative. Azide prevents growth of the organisms during shipment at ambient temperatures, thus assuring an accurate representation of the organisms present when sampled. Simultaneous with sample collection, sample data sheets were completed detailing the location where samples were collected, atmospheric conditions, and other sampling parameters. The samples were packaged and shipped via commercial carrier (see section 2.2b) to the Naval Research Laboratory (NRL), Washington, DC, for analysis. Upon arrival at NRL, the shipping package was inspected for damage. Individual samples were compared against the sample data sheets and any leaking or damaged samples were noted. The volume of each sample was recorded and the sample volume reduced. The concentrated sample was aliquoted into cryovials and archived at -80°C.

Each sample was analyzed for a variety of specific organisms. Each sample was initially screened using an antibody-based, rapid hand-held assay (8). Subsequent to the initial screening, Polymerase Chain Reaction (PCR) analysis was conducted for the sensitive detection of species specific genetic material (DNA). Data from individual assays were correlated with controls and reported using a hypertext markup language (HTML)-based report. Sample data sheets were digitized and electronically archived with the biological data from each sample. Additionally, images derived from microscopic analysis, as well as, prevailing weather patterns and satellite data maps were also archived. Encoding the report on CD-ROM provided data security with easy retrieval and accessibility upon request.

Samples were collected by the Joint Program Office (JPO-BD) using either the IBAD (Figure 1a) or the Portal Shield (Figure 1b) supported through the Advanced Concept

Technology Demonstration (ACTD) (9). Both devices are table size systems able to be located and operated remotely (Figure 1). Operation of the devices was maintained by daily irrigation of tubing. Sterile, pyrogen-free water for irrigation (USP) contained 10 % hypochlorite, and was used to wash and sanitize the collection device. Water was sent to the sampling sites to provide fluid for washing out the collection apparatus.

Figure 1. Collection devices. (A) Interim Biological Agent Detector (IBAD) and (B) Portal Shield Airbase/Port Biological Detection System

(A)
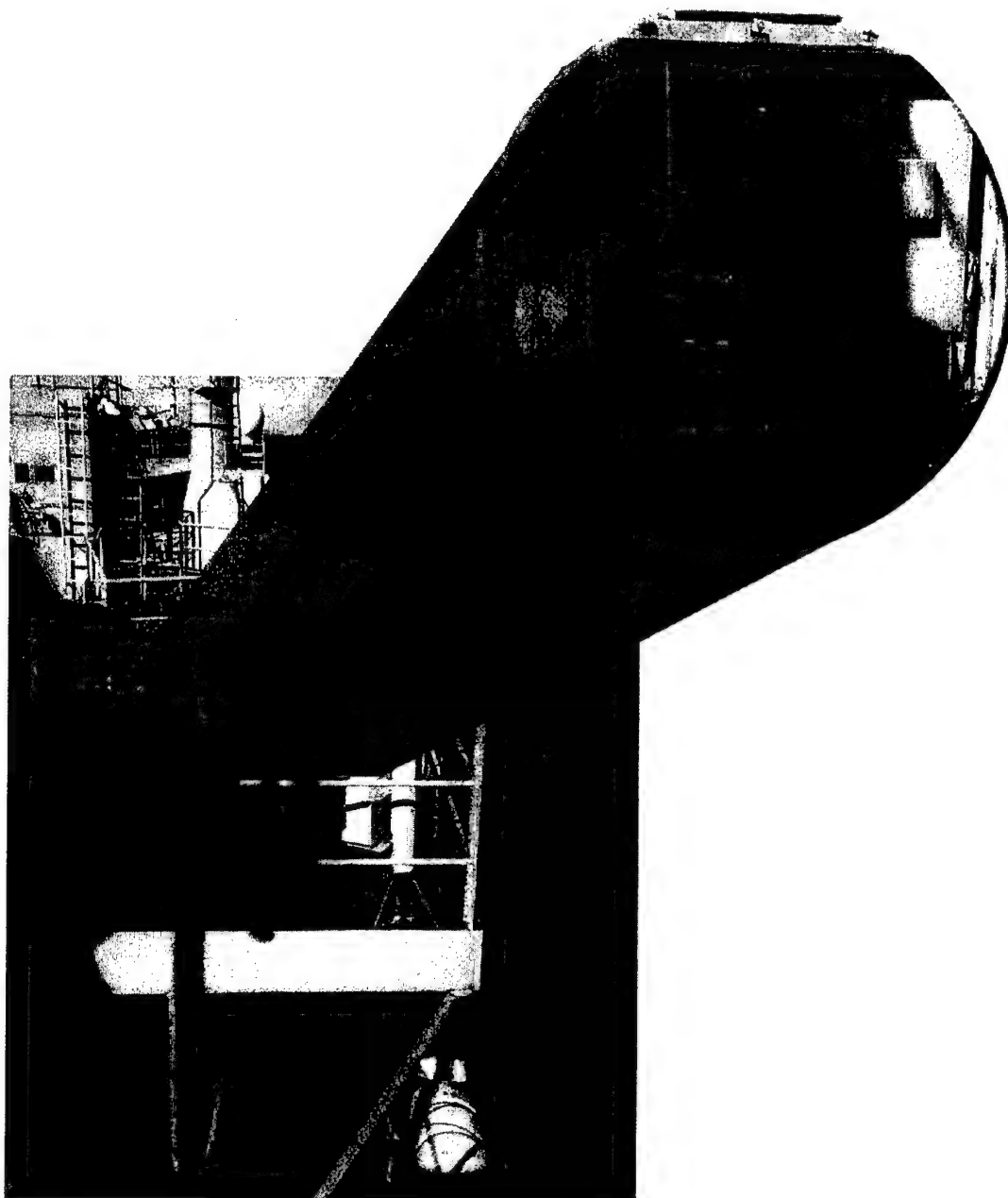
(B)
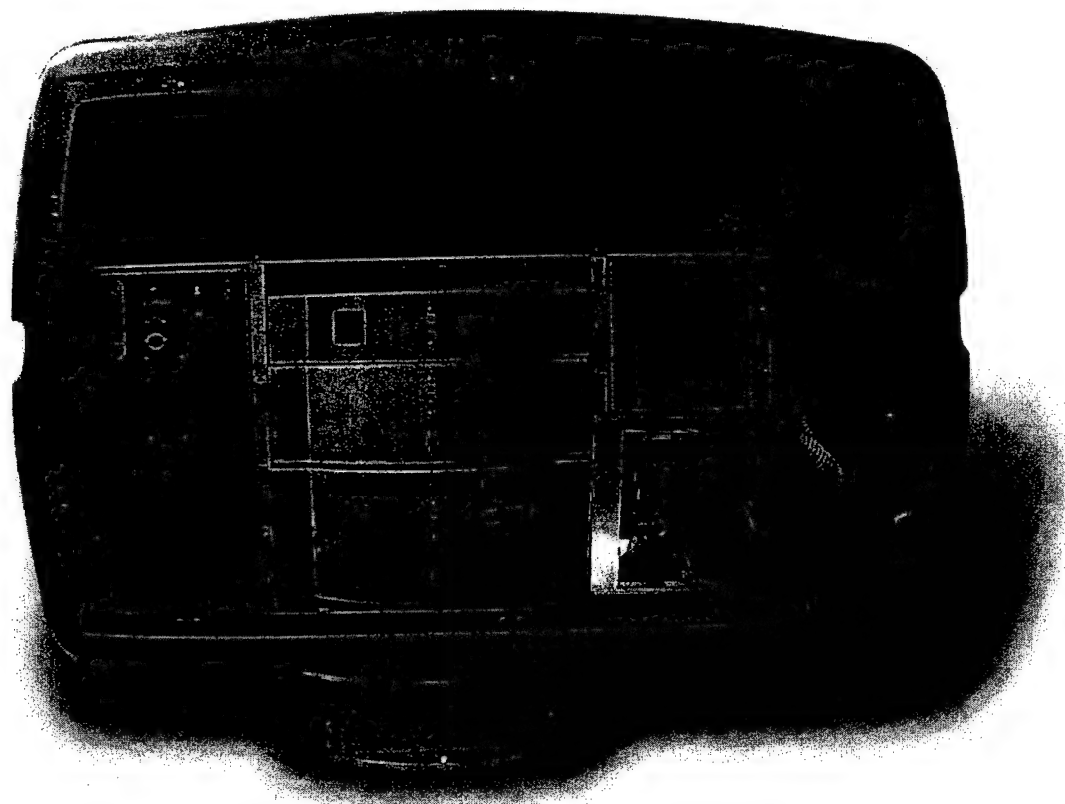
2.2 Samples

a. Sample Handling

Samples were opened in a laboratory separate from where sample analysis was conducted. All laboratory procedures were conducted in a HEPA-filtered sterile cabinet. Individually wrapped samples were removed after first breaking the "Secure-Hasp" and assessing the contents for any evidence of leaking or a compromised container (such as leaking material, color of material and overall condition of the enclosed samples). Individual sample data sheets (when present) were signed and dated at the time the accompanying sample was unpacked. Information such as date received, sample origin, condition of samples/shipping container on arrival, safety hasp number, sample identification, number of samples received for each sampling date, type of sample (i.e., collected by IBADS and Portal Shield) and collection buffer lot was also recorded. Each sample was logged, and assigned a number based on the location, date and time collected. Samples (40 ml) were concentrated by centrifugation eight-fold and aliquoted into five separate UV-treated vials. The vials were frozen and stored at -80°C until analyzed.

b. Chain of Custody

Chain of custody was maintained to monitor the integrity of the samples starting from collection to delivery to the laboratory. Transit involved passing through customs in the country where the sample was collected as well as U.S. customs. Procedures were implemented to ensure compliance and maintain custody. To help ensure integrity of the

samples during transit, the samples and fresh buffer solutions were packaged in Saf-T-Cases (Saf-T-Pak Inc., Edmonton). The security of the Saf-T-Cases was assured by using a "Secure-Hasp", a serialized one-time use hasp that confirms the package has not been opened or tampered with enroute. Samples were transported via commercial means (i.e., Federal Express or similar courier service) with completed customs documents attached to the Saf-T-Case. Total transit time averaged approximately three days.

c. Meteorological data

Meteoriological data (available on the CD version of this report) was obtained with collection using the IBAD system. Included in this data compilation are relative humidity, temperature, wind direction and wind strength. These data were recorded by hand upon sampling, and enclosed with the each sample received (on the sample data sheets). Therefore, each sample has associated meteorological data set associated with it. Additionally, GMS-5 Satellite data was obtained for each day from the Cooperative Institute for Meteorological Satellite Studies (CIMSS) at the University of Wisconsin-Madison. The GMS-5 is a spin-stabilized satellite located at 140 degree East on the geostationary orbit. The meteorological and satellite data are available on compact disk upon request to the Joint Program Office.

2.3 Laboratory Procedures

a. Buffer Preparation

Water used in production of laboratory regents was MilliQ™ UV treated. The treated water was subsequently stored for laboratory use in sterile 300 ml tissue culture flasks, or used immediately to make buffer (below). Phosphate buffered saline (PBS) 10 X (pH 7.4) was obtained from Life Technologies (Gaithersburg, MD) and diluted to 1X, supplemented with sodium azide to 0.1%(w/v) and triton X-100 to 0.1% (v/v) final concentration. Sterile Milli-Q water was used and the solutions were aliquoted into sterile Nalgene containers (500 ml capacity). The collection site was provided with sterile buffer. Separate aliquots were retained in the lab to serve as controls. Quality control testing of each buffer lot was performed by PCR to ensure no contamination of the buffer by DNA sequences had occurred.

b. DNA Preparation

Individual samples representing 20 separate hours within a particular sampling day, were pooled prior to laboratory analysis. Once pooled, the samples were extracted as described (10). Recovered nucleic acids were resuspended in MilliQ water, and stored at 4°C. This partially purified nucleic acid prep was used as the substrate for further analysis by polymerase chain reaction for three pathogens (*Bacillus anthracis*, *Yersinia pestis* and *Francisella tularemia*). The pooled samples were also used in procedures where amplification of bacterial 16S ribosomal genes was conducted by polymerase chain reaction using primers complementary to conserved regions of this gene. Primers were designed such that 16S genes would be amplified from most bacterial species regardless of genetic relatedness.

c. Polymerase Chain Reaction (PCR)

Either of two PCR assays procedures was utilized in analyzing all samples. In the first method, PCR specific for a single agent was conducted for the detection of either *B. anthracis*, *Y. pestis* or *F. tularensis*. These assays were developed at the Naval Medical Research Institute in Bethesda, MD (currently Naval Medical Research Center, Forest Glen, MD). The second PCR assay was a "triplex" assay developed at NRL. The triplex assay was designed to specifically detect three bacterial pathogens simultaneously: *B. anthracis*, *F. tularensis*, and *Y. pestis*. PCR detection of *B. anthracis*, was designed to detect capsule gene A (CapA), protective antigen (PA) or both. For specific PCR detection of *Y. pestis*, a single primer set was utilized, which was designed to amplify the chromosomally located plasminogen activator gene. Similarly, for specific PCR detection of *F. tularensis*, the causative agent of tularemia, primers were designed to amplify the chromosomally located TUL4 gene, which encodes an outer membrane protein that is highly conserved in clinical isolates. The primers were designed, using the PRIME software program (available as part of the Wisconsin Package™, version 9; Genetics Computer Group, Madison, WI), such that they could be added together in a single assay, each detecting a specific organism without cross interference. After amplification, amplicons were differentiated by agarose gel electrophoresis.

d. Bacterial Identification by 16S Sequencing

The objective of the study was to evaluate the microbiological flora of specific regions. Because of the plethora of bacterial genera and species potentially present, it was not possible to analyze, for every potential bacterial genera and species likely to exist in a sample. Furthermore, because of the scope of organisms likely present in the selected geographic area, rapid molecular or biochemical assays would not be available for most bacteria represented in the collected samples.

Not withstanding the lack of specific assays available, it is important to identify, as closely as possible, as many bacterial genera/species as possible. Furthermore, the analysis method selected must be capable of accurate evaluation, be capable of standardization, be relatively inexpensive, rapid and automatable because of the large number of samples to be analyzed. Because of the inherent caveats to any biochemical detection regime, DNA sequences of amplified 16S ribosomal DNA present in the pooled samples was compared. 16S ribosomal gene sequences are highly conserved within bacteria and well represented in the published genetic databases. Therefore, comparison of 16S sequences can lead to identification of genera and, in many cases, species for bacteria from environmental samples.

Total nucleic acid was extracted from each pooled sample and was subjected to PCR amplification of the 16S gene. For amplification, 10% extracted DNA was suspended in 10 mM Tris-HCl, pH 8.3, 50 mM KCl with 100 pMol of 16S specific DNA primers (Bioserve Biotechnologies Ltd., Laurel, MD). The forward primer was 17 bases and the reverse primer was 22 bases in length, hybridizing the 16S gene at bp 340-357 (5' – CCT ACG GGA GGC AGC AG – 3') and 928-907 (5' – CCC CGT CAA TTC CTT TGA GTT T – 3'), respectively (*E. coli* number system)(11). To each PCR sample set, 400 μM each dNTP, 2.5 mM MgCl$_2$, 5% (v/v) DMSO and 2.5 U AmpliTaq LD/ TaqStart mixture was added. The samples were heated to 92° C for 5 min. followed by forty cycles of the following profile: 92°C for 30 sec., anneal at 55°C for 30 sec. and extend at 72°C for 1 min.

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Following amplification, 7.5 μl of PCR product was run on a 1 % (w/v) tris borate EDTA TBE agarose gel to confirm production of amplified product. After confirmation by electrophoresis, positive amplicons were cloned, using the TOPO-TA (Invitrogen, Carlsbad, CA) vectors system according to the manufacturer's directions. Five to ten recombinant (white) colonies were picked at random and analyzed for the presence of inserts (Applied Biosystems, Foster City, CA). The resulting plasmids were cycle sequenced using Big Dye (Applied Biosystems, Foster City, CA) terminator chemistry and the ABI 310 Genetic Analyzer (Applied Biosystems). Sequences derived from this analysis were compared to the "small ribosome subunit database" using BLAST (12). Species was determined by comparing resulting sequence information to published databases (Genbank) for 16S genes.

Data obtained from BLAST searches include Expect (E) values. A statistical value is used to report the significance of matches within a database. The E value decreases exponentially with the Score (S) that is assigned to a match between two sequences. An E value of 1 assigned to a hit can be interpreted as meaning that in the 16S database of the current size (at the National Library of Medicine) one might expect to see one match with a similar score simply by chance.

3.0 TECHNOLOGY IMPLEMENTATION

3.1 DoD Need

Although The Biological Warfare Conference (BWC) entered into force in 1975 prohibits the development, production, stockpiling and acquisition of biological weapons; there is no provision for enforcement or transparency. In 1998, the President of the United States called for strengthening the BWC. Completion of this, however, requires adequate methods to control and monitor biological agent use.

Pathogenic bacteria and viruses, including those used as biowarfare agents, exist throughout the world. Therefore, in order to ensure compliance of rogue states with the BWC and to ensure usage or exposure to biowarfare agents of troops stationed in strategic geographic locations, an understanding of the normal endemic microbiologic flora is necessary to in order to begin to establish the normal background microbiology. The purpose of this study was to obtain yearlong data capable of establishing background levels of bacteria in strategically important areas.

3.2 Summary/Transition

Samples from overseas air collection were obtained and sent to NRL for detailed analysis. 16S rDNA, present in the samples was amplified and sequenced. Data was obtained in the form of 16S rDNA sequence comparison with known sequences from bacteria published in the publicly available Genebank. A brief compilation of that data is reported here. However, further, raw data, is available in compact disk (CD) format, through the Joint Program Office. This data contains raw match scores from Genebank, meteorological data and satellite information at the time particular the samples were collected. Knowledge of the normal bacterial background, throughout the year, will be critical for future assessments of bioagent use in these regions.

4.0 LESSONS LEARNED

4.1 Sequences and Perspective

The objective of this study was to identify the myriad genera of bacteria (culturable or non-culturable) endemic in air samples from strategically important regions of the world using rapid, sensitive techniques. Genetic techniques were selected in order to avoid skewing results by over-reporting due to bias inherent in culture adaptability of some organisms over others or bias inherent in some specific molecular biology techniques. In light of this requirement, a comparison of DNA sequences from amplified and cloned 16S ribosomal genes was conducted.

PCR primers were chosen to amplify the greatest number of organisms by virtue of the highly conserved of regions of the 16S rRNA gene in eubacteria. Amplicons resulting from the PCR using mixed organisms should, with minimal bias, contain an accurate representation of bacterial 16S rRNA sequence. Consequently, comparison of sequences to published databases should yield a relatively complete assessment of the predominant bacterial genera species collected in the air samples. This data will be highly valuable in future assessment of deliberate, biological agent use in the area.

4.2 Potential Error

Error can be introduced into the scheme at a number of points. First, early in the collection it was noticed that growth of a biofilm existed in the tubing of the collectors. Although these bacteria were likely to be representative of endemic organisms, they would be present and potentially introduced into the samples at unusually high levels. This was easily corrected by prewashing the collection tubing with a 10% bleach solution followed by a sterile water rinse prior to actual sample collection.

Additionally, error could have been introduced into the samples by human contact during the collection and packaging process. Because PCR is an extremely sensitive technique, small amounts of contaminating DNA can easily be detected and may confound the data. During the course of this study, sample vials were handled with gloved hands and extreme care was taken in the preparation and packaging of solutions including the buffers used. In the laboratory, PCR reagents were physically separated in different laboratories from where the opened samples were handled. Furthermore, PCR assay "runs" were consistently monitored with controls to detect contaminated reagents.

Error introduction may also occur by erroneous over-representation at the PCR and cloning steps. However, any error at these steps would be minimized for a number of reasons. Primers and target genes selected for use in PCR were based on amplification of the relatively ubiquitous and conserved 16S rRNA gene. Thus amplification efficiencies should be comparable from bacteria to bacteria. However, some error is possible if a given bacteria contains divergent and interfering non-conserved regions of the ribosomal RNA gene. However, this is unlikely except in very rare cases. Another potential point of error exists at the cloning stage, since the genes selected for final sequence determination are predicated on growth of *E. coli* containing the inserted DNA. However, the likely error introduced at this point is low. The inserts in each cloning are the same size (within one or two base pairs). Therefore, growth of clones containing any particular insert will be comparable to inserts in

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any other clone. Thus, there was no selective pressure predicated on size of the DNA insert for one clone to grow faster than another. This point was evident by the relatively uniform size of *E. coli* clones on agar plates. The colonies containing inserts were randomly selected from the culture plates for sequencing of insert. Five to ten colonies were selected for sequencing per sample, further reduced the likely-hood of missing highly represented bacteria. Despite the above, some endemic bacterial species represented at very low amounts in the samples may not have been cloned (i.e., under-represented). Conversely, it is possible bacterial species that were present in very low amounts in the air-samples were, on the rare occasion, expressed and cloned at an unusually high rate (i.e., over-reported).

4.3 Technology Effectiveness and Usefulness

This study was designed not to assess the epidemiologic distribution of organisms but rather to evaluate aerosolized microflora in strategic sections of the World. These data, in tandem with follow-on studies, will be useful in the: (1) design of prophylactic treatment choices in deploying troops; and (2) determination of deliberate use of biological agents.

In addition to the data collected, it was important to develop generic methodologies for the collection and identification of microorganisms over an extended period of time. In order to fulfill this need, developed methods must be standardizable, relatively automatable (i.e., relatively low labor intensive) and be capable of accurate evaluation and identification of bacteria. Results reported here indicate that the methodology used can be an extremely effective regimen for obtaining long-term data. The completeness of the data is driven by the sample collection steps. In this study, samples were only collected by air sampler. In retrospect, samples collected from soil and surrounding water supplies should have been collected to give a more complete evaluation of the endemic flora.

However, notwithstanding the study caveats, the results of this study alone and in conjunction with similar datasets can be helpful in not only determining the deliberate release of biological agents but also help in the evaluation of emerging diseases globally. With increased rapid travel around the World in concert with increased encroachment by humans into new geographic areas, many organism not previously regarded as pathogenic are beginning to emerge as serious health issues. Continuous, long-term evaluation of microflora Worldwide with ready sharing of results will be instrumental in detecting potential health effects prior to the beginning of epidemics.

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Appendix A

OSAN, KOREA

Air samples were collected in Osan, Korea by either IBADS or the Portal Shield/Port Biological Detection Collection system, from October 1997 to July 1998. From Osan, we received a total of 989 samples. This represents thirty-three separate sampling days over a nine-month period (collections on the IBADs were approximately every hour for 20 hours per day). The sample dates, sample identifier and number of samples collected on each date are listed in Table 1.

RESULTS

Hourly samples collected on a specific day were pooled to give a daily sample. After pooling, daily samples were subjected to PCR amplification of 16S rDNA. In some cases, that day's sample was also subjected to PCR amplification for specific detection of *B. anthracis, F. tularemia and/or Y. pestis*, as described in section 2.3.

Table 2 (panels A – hh) shows the results of PCR amplification and subsequent sequence comparison of amplicon with Genebank database for best fit in order to determine the bacterial origin of the sequenced gene. Each number, as in Table 1, corresponds to the collection location (KO: Korea, Osan) and the date of collection (i.e., 031097 was collected 3 October 1997). Shown are the best fit for each of 5 - 10 colonies picked for subsequent sequencing. The column, "Identity", is the organism with the highest score in the database comparison (best sequence match). The column labeled, "Clinical significance", gives a brief descriptive comment of identified microorganism.

In addition to amplification and sequencing of the bacterial 16S rDNA for bacterial identification, samples from each day were subjected to analysis by PCR using agent specific primers. PCR assays specific for *B. anthracis, Francisella tularensis and Yersinia pestis* were conducted, as described previously. It is important to note that none of these bacteria were detected in any samples analyzed.

Detailed Genebank results with statistical scores are available through the Joint Program Office as compact disks. Also, as previously mentioned, meteorological data collected at the time samples were collected is also available through the Joint Program Office.

Detected Microflora

A number of bacterial agents have been implicated to be either in development as biological warfare agents by rogue states or have been developed (13). Of primary importance is the use of *Bacillus anthracis*, the causative agent of anthrax due to its lethality and viability even under harsh environmental conditions. Because of the significant threat of use of this agent a great deal of focus, internationally, has been placed on detection of this agent.

Inspection of the "Clinical Significance" column of Table 2 suggests many of the organisms found are pathogens. This is not the case. With the exception of the *F. tularensis*, most of these organisms are normal soil inhabitants, and do no normally cause infection. Microorganisms such as *Methylobacterium, Propionibacterium, Ralstonia, Staphylococcus, Clostridium, Actinomycete,* and *Bacteroides* all occur normally in the environment. It is only when they are able to penetrate the skin, normally by trauma, that they are capable of infecting humans. Normal sanitary measures such as hand washing, and routine wound cleaning are normally enough to avoid infections with these micro-organisms.

The genus *Bacillus* includes many different species of bacteria, although they all share common morphological and physiological traits. However, all members of this genus are Gram-positive rods found primarily as soil saprophytes (many species are found world-wide). As a group, they all form endospores in the presence of specific environmental conditions and are thus likely to be represented in high numbers in environmental samples, worldwide. *Bacillus* spp. have a relatively thick cell wall and are resistant to physical disruption, such as osmotic shock. Therefore, members of this genera are very hardy and are able to survive deleterious conditions (i.e., droughts, nutrient limitations, osmotic shock, etc).

Within the *Bacillus* genus, several distinct groups exist based on relatedness of 16S rRNA sequences. In fact, the rRNA sequence of *Bacillus cereus* varies from that of *Bacillus anthracis* by only one base over a total of 1544 bases. Therefore, it is sometimes difficult to actually identify to the species level in the genus *Bacillus* based only on 16S rRNA sequence. Nevertheless, the computer software used to compare sequence data (BLAST) often gives suggested species based on "best fit." However, since virulence of *B. anthracis* resides in the expression of specific gene products (i.e., lethal factor, edema factor, protective antigen, as well as capsular genes, encoded on two relatively small plasmids) confirmation of identity of *B. anthracis* is easily accomplished by analysis for the presence of these genes or gene products (14). Because of the importance of this organism, throughout our sampling events, in addition to 16S rRNA evaluation, specific PCR analysis was carried out in order to confirm the presence *Bacillus anthracis* virulence genes. Also, specific PCR analysis was done to screen for Y. *pestis* using primers specific for the plasminogen activator gene and for *F. tularemia* sung TUL 4 gene primers. In the Osan samples, neither *B.* anthracis, *Y. pestis* or *F. tularemia* were detected by either 16S rRNA sequencing or specific PCR.

The results of all samples analyzed are given in Table 2. Presented in Table 2 is the best match from the Genebank comparison. In some cases, sequences of the clone did not result in an identity of the bacterial origin. This is not surprising since the database used, although constantly being improved, is still not complete for all potential bacterial genera and species. However, other clones did result in scores yielding a bacterial match. For those bacteria that were identified, relatively few were validated threat agents. The low numbers of recognized pathogenic agents is possibly due to low levels of aerosolization of bacteria when the samples were collected.

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Table 1. Air samples collected in Osan, Korea for 20 consecutive hours

Sample Date	Sample Identifier	IBADS	Portal Shield
15 Aug 1997	KO150897	2	
03 Nov 1997	KO031197	20	121
07 Nov 1997	KO071197		133
20 Nov 1997	KO201197	20	
30 Nov 1997	KO301197	21	7
13 Dec 1997	KO131297	20	
27 Dec 1997	KO271297	21	38
28 Dec 1997	KO281297	20	
01 Jan 1998	KO010198	20	
02 Jan 1998	KO020198	20	
16 Jan 1998	KO160198	18	
20 Jan 1998	KO200198		73
29 Jan 1998	KO290198	20	
11 Feb 1998	KO110298	20	
27 Feb 1998	KO270298	20	
09 Mar 1998	KO090398	20	
01 Mar 1998	KO010398	18	
16 Mar 1998	KO160398	20	
20 Mar 1998	KO200398	20	
05 Apr 1998	KO050498	18	
07 Apr 1998	KO070498	18	
16 Apr 1998	KO160498	20	
25 Apr 1998	KO250498	20	
03 May 1998	KO030598	20	
05 May 1998	KO050598	20	
10 May 1998	KO100598	20	
30 May 1998	KO300598	21	22
10 Jun 1998	KO100698	18	
23 Jun 1998	KO230698	20	
27 Jun 1998	KO270698	20	
07 Jul 1998	KO070798	20	
16 Jul 1998	KO160798	20	
26 Jul 1998	KO260798	20	
	Totals	595	394

Table 2. 16S rDNA Sequencing

A.

KO 150897 (15 August 1997)

Colony	Identity	Clinical Significance
1	*Staphylococcus equorum*	Non-pathogenic in humans
2, 3	Unidentified	Unknown
4, 5	*Afipia* spp.	Non-pathogenic in humans

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B.

KO 031197 (3 November 1997)

Colony	Identity	Clinical Significance
1	*P. prevotii*	Non-pathogenic in humans
2	*Leptothrix* spp.	Non-pathogenic in humans
3	*Chondromyces robustus*	Non-pathogenic in humans
4	Unidentified	Unknown
5	Enterococus faecium	Found in GI of animals and humans and possibly urinary tract; potentially pathogenic.

C.

KO 071197 (7 November 1997)

Colony	Identity	Clinical Significance
1	*Clostridium methylpentosum*	Presumptively non-pathogenic
2	*Agrobacterium* spp.	Non-pathogenic in humans
3	Unidentified	Unknown
4	Beta-proteobacterium	Unknown
5	*Nocardioides* spp.	Infection in humans rare except for immunocompromised; some species cause skin abscess

D.

KO 201197 (20 November 1997)

Colony	Identity	Clinical Significance
1, 2, 5	*Arabidopsis thaliana*	Non-pathogenic in humans
3	*Cyanobacterium* spp.	Non-pathogenic in humans
4	*Treponema* spp.	Species cause disease in humans such as syphilis, yaws and pinta

E.

KO 301197 (30 November 1997)

Colony	Identity	Clinical Significance
1, 5	Unidentified	Unknown
2	*Staphylococcus lentus*	Not known to be pathogenic in humans
3	*Neisseria perflava*	Not known to be pathogenic in humans
4	*Lactobacillus gasseri*	Not known to be pathogenic in humans

F.

KO 131297 (13 December 1997)

Colony	Identity	Clinical Significance
1, 3	*Clostridium difficile*	Causes diarrhea in humans
2	*Nicotiana tabacum*	Not known to be pathogenic in humans
4	*Variovaorax* spp.	Not known to be pathogenic in humans
5	*Azospirillum amazonense*	Not known to be pathogenic in humans

G.

KO 271297 (27 December 1997)

Colony	Identity	Clinical Significance
1	*Thermomicrobium roseum*	Not known to be pathogenic in humans
2	Unidentified	Unknown
3	*Bacteroides* spp.	Some species are important anaerobic bacteria causing diarrhea and abdominal infections
4	*Brachybacterium tyrofermentans*	Not known to be pathogenic in humans
5	*Deinococcus proteolyticus*	Not known to be pathogenic in humans

H.

KO 281297 (28 December 1997)

Colony	Identity	Clinical Significance
1,3	uncultured bacterium	Related to rumen bacteria; organisms found in cattle intestines
2	unidentified eubacterium	Related to *Rhizobium*, another soil organism
4	*Lactobacillus gasseri*	Non pathogenic
5	unidentified bacterium	Related to *Agrobacterium*, a common soil organism

I.

KO 010198 (1 January 1998)

Colony	Identity	Clinical Significance
1	uncultured bacterium	Related to soil *Actinomycete*, normal soil flora.
2	unknown *Actinomycete*	Related to soil *Actinomycete*, normal soil flora.
3, 5	uncultured bacterium	Related to rumen bacteria; organisms found in cattle intestines.
4	*Clostridium glycolicum*	Found in the rumens of cattle, and digestive tracts of humans. Not uncommon in soil.

J.

KO 020198 (2 January 1998)

Colony	Identity	Clinical Significance
1, 5	*Arthrobacter* sp.	Not known to be pathogenic in humans
2	Uncultured bacterium	Related to *Bacillus*. Not differentiated from some *Bacillus* pathogens
3	Cloning vector pTacSfi	Error in cloning/ sequencing
4	Unidentified bacterium	Unknown

K.

KO 160198 (6 January 1998)

Colony	Identity	Clinical Significance
1	*Janthinobacterium lividum*	Not known to be pathogenic in humans
2	*Chondromyces lanuginosus*	Not known to be pathogenic in humans
3	Uncultured rumen bacterium	Related to rumen bacteria; organisms found in cattle intestines.
4	Unidentified eubacterium	Found in the rumens of cattle, and digestive tracts of humans. Not uncommon in soil.
5	*Clavibacter michiganensis*	Not known to be pathogenic in humans

L.

KO 200198 (20 January 1998)

Colony	Identity	Clinical Significance
1	Uncultured bacterium	Unknown
2	*Nocardioides jensenii*	Saprophytic soil organism, responsible for decomposing organism plant matter.
3, 4, 5	Uncultured bacterium	Presumptively non-pathogenic

M.

KO 290198 (29 January 1998)

Colony	Identity	Clinical Significance
1, 3	Manganese-oxidizing bacterium	Not known to be pathogenic in humans
2, 5	Unknown bacterium	Presumptively non-pathogenic
4	*Rhodococcus* sp.	Not known to be pathogenic in humans

N.

KO 110298 (11 February 1998)

Colony	Identity	Clinical Significance
1	Uncultured bacterium	Related to rumen bacteria; organisms found in cattle intestines.
2	*Chlorella mirabilis*	DNA from Plant Chloroplast
3	*Clostridium disporicum*	Presumptively non-pathogenic
4	*Hyphomicrobium hollandicum*	Presumptively non-pathogenic. Often found in animal feces.
5	Unidentified rumen bacterium	Related to rumen bacteria; organisms found in cattle intestines.

O.

KO 270298 (27 February 1998)

Colony	Identity	Clinical Significance
1	Unidentified soil eubacterium	Related to *Clostridium* species. Presumptively non-pathogenic
2	No match	Error in cloning/sequencing
3	Unidentified rumen bacterium	Presumptively non-pathogenic
4	Unidentified rumen bacterium	Presumptively non-pathogenic
5	Uncultured bacterium	Presumptively non-pathogenic

P.

KO 010398 (1 March 1998)

Colony	Identity	Clinical Significance
1, 4	Uncultured bacterium	Presumptively non-pathogenic
2	*Staphylococcus lentus*	Non-pathogenic in humans
3, 5	Uncultured bacterium	Unknown

Q.

KO 090398 (9 March 1998)

Colony	Identity	Clinical Significance
1	*Variovorax* sp.	Presumptively non-pathogenic
2	Unidentified eubacterium	Unknown
3	Uncultured bacterium	Unknown
4	Bacterium strain	Unknown
5	Metal-contaminated soil clone	Related to soil bacteria, presumptively non-pathogenic

R.

KO 160398 (16 March 1998)

Colony	Identity	Clinical Significance
1	Uncultured cyanobacterium	Presumptively non-pathogenic
2	*Flavobacterium ferrugineum*	Non-pathogenic
3	*Lactobacillus crispatus*	Presumptively non-pathogenic
4	*Staphylococcus gallinarum*	Presumptively non-pathogenic
5	*Zea mays* chloroplast	Non-pathogenic

S.

KO 200398 (20 March 1998)

Colony	Identity	Clinical Significance
1, 2	Unidentified bacterium	Presumptively non-pathogenic
3	Uncultured marine Eubacterium	Unknown
4, 5	Uncultured rumen bacterium	Unknown; related to rumen bacteria (micro-organisms found in cattle intestines)

T.

KO 050498 (5 April 1998)

Colony	Identity	Clinical Significance
1	*Burkholderia caryophylli*	Plant pathogen, but normal soil inhabitant
2	*Rubrivivax gelatinosus*	Non-pathogenic
3	*Methylobacterium* sp.	Non-pathogenic
4	*Prevotella dentalis*	Non-pathogenic
5	Uncultured bacterium	Presumptively non-pathogenic
6	*Ralstonia* sp.	Non-pathogenic
7	*Propionibacterium acnes*	Causes acne vulgaris
8	Unidentified rumen bacterium	Unknown
9, 10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample

U.

KO 070498 (7 April 1998)

Colony	Identity	Clinical Significance
1	Unidentified soil eubacterium	Unknown
2	Uncultured bacterium	Unknown
3	*Ruminococcus albus*	Non-pathogenic
4	*Clostridium disporicum*	Presumptively non-pathogenic
5	*Terrabacter* sp.	Non-pathogenic

V.

KO 160498 (16 April 1998)

Colony	Identity	Clinical Significance
1	*Nicotiana plumbaginifolia* chloroplast	None
2	Gamma proteobacterium	Not known to be pathogenic in humans
3, 5	Unidentified eubacterium	Unknown
4	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample

W.

KO 250498 (25 April 1998)

Colony	Identity	Clinical Significance
1	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
2	*Nicotiana tabacum* chloroplast	None
3	Uncultured eubacterium	Presumably non-pathogenic
4	*Bacillus subtilis*	Not known to be pathogenic in humans
5	*Alkalispirillum mobilis*	Presumably non-pathogenic
6	*Sphenostylis stenocarpa*	Presumably non-pathogenic
7	*Juniperus virginiana* chloroplast	None
8	*Brachybacterium alimentarium*	Presumably non-pathogenic
9	*Nicotiana plumbaginifolia* chloroplast	None
10	*Paracoccus* sp.	Presumably non-pathogenic

X.

KO 030598 (3 May 1998)

Colony	Identity	Clinical Significance
1, 2, 3, 6, 7, 9, 10	*Pinus thunbergii* chloroplast	None
4	*Lactosphaera pasteurii*	Presumably non-pathogenic
7	*Pinus thunbergii* chloroplast	None
8	*Nicotiana plumbaginifolia* chloroplast	None

Y.

KO 050598 (5 May 1998)

Colony	Identity	Clinical Significance
1	*Pinus thunbergii* chloroplast	None
2	Unidentified eubacterium clone	Presumably non-pathogenic
3	*Rubromicrobium aerophilum*	Presumably non-pathogenic
4	*Caulobacter* sp.	None
5	Unidentified eubacterium	Presumably non-pathogenic

Z.

KO 100598 (10 May 1998)

Colony	Identity	Clinical Significance
1, 2	Bacterium strain	Presumably non-pathogenic
3, 4, 5	*Variovorax* sp.	Presumably non-pathogenic

aa.

KO 300598 (30 May 1998)

Colony	Identity	Clinical Significance
1, 4, 5	*Propionibacterium acnes*	Causes acne vulgaris or acne of the skin
2, 3	Unidentified beta proteobacterium	Presumably non-pathogenic

bb.

KO 100698 (10 June 1998)

Colony	Identity	Clinical Significance
1,2,5,6,8-10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample.
	Bacillus subtilis	Non-pathogenic
	Bacillus subtilis	Non-pathogenic
	Sulfobacillus sp.	Non-pathogenic

cc.

KO 230698 (23 June 1998)

Colony	Identity	Clinical Significance
1, 6	*Sulfobacillus* sp.	Non-pathogenic
2	Unidentified low G+C Gram-positive bacterium	Presumably non-pathogenic
3, 4, 8, 10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
5	*Paenibacillus amylolyticus*	Presumably non-pathogenic
7	*Bacillus subtilis*	Non-pathogenic
9	*Brevibacillus choshinensis*	Presumably non-pathogenic

dd.

KO 270698 (27 June 1998)

Colony	Identity	Clinical Significance
1	*Renibacterium almoninarum*	Presumably non-pathogenic
2, 3, 4, 5	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
6	*Brevibacillus choshinensis*	Non-pathogenic
7	*Bacillus sphaericus*	Non-pathogenic
8, 9, 10	*Bacillus subtilis*	Non-pathogenic

ee.

KO 070798 (7 July 1998)

Colony	Identity	Clinical Significance
1, 5, 6, 10	*Bacillus subtilis*	Non-pathogenic
2	*Bacillus pseudofirmus*	Non-pathogenic
3, 4, 9	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
7	*Paenibacillus amylolyticus*	Non-pathogenic
8	*Renibacterium almoninarum*	Non-pathogenic

ff.

KO 160798 (16 July 1998)

Colony	Identity	Clinical Significance
1, 6, 7	*Bacillus subtilis*	Non-pathogenic
2	*Tissierella praeacuta*	Non-pathogenic
3-5, 8-10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample

gg.

KO270798 (27 July 1998)

Colony	Identity	Clinical Significance
1, 10	*Methylobacterium* species	Non-pathogenic
2	Unidentified rumen bacterium	Presumably non-pathogenic
3-5, 8, 9	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
6	*Methylobacterium extorquens*	Non-pathogenic
7	Unidentified bacterium	Unknown

Appendix B

KUNSAN, KOREA

Air samples were collected in Kunsan, Korea by either IBADS or the Portal Shield/Port Biological Detection Collection system, from October 1997 to July 1998. From Kunsan, we received a total of 565 samples. This represents twenty-seven separate sampling days over a nine-month period (collections on the IBADs were approximately every hour for 20 hours per day). The sample dates, sample identifier and number of samples collected on each date are listed in Table 1.

RESULTS

Hourly samples collected on a specific day were pooled to give a daily sample. After pooling, daily samples were subjected to PCR amplification of 16S rDNA. In some cases, that day's sample was also subjected to PCR amplification for specific detection of *B. anthracis, F. tularemia and/or Y. pestis*, as described in section 2.3.

Table 2 (panels A – hh) shows the results of PCR amplification and subsequent sequence comparison of amplicon with Genebank database for best fit in order to determine the bacterial origin of the sequenced gene. Each number, as in Table 1, corresponds to the collection location (KK: Korea, Kunsan) and the date of collection (i.e., 031097 was collected 3 October 1997). Shown are the best fit for each of 5 - 10 colonies picked for subsequent sequencing. The column, "Identity", is the organism with the highest score in the database comparison (best sequence match). The column labeled, "Clinical significance", gives a brief descriptive comment of identified microorganism.

In addition to amplification and sequencing of the bacterial 16S rDNA for bacterial identification, samples from each day were subjected to analysis by PCR using agent specific primers. PCR assays specific for *B. anthracis, F. tularensis and Y. pestis* were conducted, as described previously. The analysis of 6 July, 1998 was positive for *F. tularensis*. However, the same sample was used for the 16S sequencing, where it was not identified. This is consistent with the organism being present at very low levels.

Detected Microflora

A number of bacterial agents have been implicated to be either in development as biological warfare agents by rogue states or have been developed (13). Of primary importance is the use of *B. anthracis*, the causative agent of anthrax due to its lethality and viability even under harsh environmental conditions. Because of the significant threat of use of this agent a great deal of focus, internationally, has been placed on detection of this agent.

Examination of the organisms identified reveals only two organisms that would be considered a biological agents: *F. tularensis* identified in the 12 May sample, and *Y. pestis* identified in the 30 April sample. As discussed above, it is unlikely this is

evidence of a biological attack, but the detection level of these systems are low enough to detect organisms which are present in the environment. Inspection of the "Clinical Significance" column of Table 2 suggests many of the organisms found are pathogens. This is not the case. With the exception of the *F. tularensis*, most of these organisms are normal soil inhabitants, and do no normally cause infection. Microorganisms such as *Paenibacillus, Clostridium, Brevibacillus, Acidovorax, Methylobacterium, Streptococcus, Globicatella, Eubacterium, Propionibacterium, Lactobacillus, Comamonas, Sphingomonas* all occur normally in the environment. It is only when they are able to penetrate the skin, normally by trauma, that they are capable of infecting humans. Normal sanitary measures such as hand washing, and routine wound cleaning are normally enough to avoid infections with these microorganisms.

The genus *Bacillus* includes many different species of bacteria, although they all share common morphological and physiological traits. However, all members of this genus are Gram-positive rods found primarily as soil saprophytes (many species are found world-wide). As a group, they all form endospores in the presence of specific environmental conditions and are thus likely to be represented in high numbers in environmental samples, worldwide. *Bacillus* spp. have a relatively thick cell wall and are resist to physical disruption, such as osmotic shock. Therefore, members of this genera are very hardy and are able to survive deleterious conditions (i.e., droughts, nutrient limitations, osmotic shock, etc).

Within the *Bacillus* genus, several distinct groups exist based on relatedness of 16S rRNA sequences. In fact, the rRNA sequence of *Bacillus cereus* varies from that of *Bacillus anthracis* by only one base over a total of 1544 bases. Therefore, it is sometimes difficult to actually identify to the species level in the genus *Bacillus* based only on 16S rRNA sequence. Nevertheless, the computer software used to compare sequence data (BLAST) often gives suggested species based on "best fit." However, since virulence of *B. anthracis* resides in the expression of specific gene products (i.e., lethal factor, edema factor, protective antigen, as well as capsular genes, encoded on two relatively small plasmids) confirmation of identity of *B. anthracis* is easily accomplished by analysis for the presence of these genes or gene products (14). Because of the importance of this organism, throughout our sampling events, in addition to 16S rRNA evaluation, specific PCR analysis was carried out in order to confirm the presence *Bacillus anthracis* virulence genes. In addition, specific PCR analysis was done to screen for *Y. pestis* using primers specific for the plasminogen activator gene and for *F. tularemia* sung TUL 4 gene primers. In the Kunsan samples, neither *B.* anthracis, *Y. pestis* or *F. tularemia* were detected by either 16S rRNA sequencing or specific PCR.

The results of all samples analyzed are given in Table 2. Presented in Table 2 is the best match from the Genebank comparison. In some cases, sequences of the clone did not result in an identity of the bacterial origin. This is not surprising since the database used, although constantly being improved, is still not complete for all potential bacterial genera and species. However, other clones did result in scores yielding a bacterial match. For those bacteria that were identified, relatively few were validated threat agents. The

low numbers of recognized pathogenic agents is possibly due to low levels of aerosolization of bacteria when the samples were collected.

Table 1. Air samples collected in Kunsan, Korea for 20 consecutive hours

Sample Date	Sample Identifier	IBADS	Portal Shield
14 Oct 1997	KK141097	20	
19 Oct 1997	KK191097	20	
10 Nov 1997	KK101197	20	
14 Nov 1997	KK141197	20	
22 Nov 1997	KK221197	20	
20 Dec 1997	KK201297	20	
04 Jan 1998	KK040198	20	
23 Jan 1998	KK230198	20	
27 Jan 1998	KK270198	20	
04 Feb 1998	KK040298	20	
13 Feb 1998	KK130298	20	
18 Feb 1998	KK180298	20	
10 Mar 1998	KK100398	20	
12 Mar 1998	KK120398	20	
17 Mar 1998	KK170398	20	
21 Apr 1998	KK210498	20	
23 Apr 1998	KK230498	20	
30 Apr 1998	KK300498	20	
12 May 1998	KK120598	20	
14 May 1998	KK140598	20	
17 May 1998	KK170598	20	
31 May 1998	KK310598	20	
18 Jun 1998	KK180698	20	5
30 Jun 1998	KK300698	20	5
06 Jul 1998	KK060798	20	5
14 Jul 1998	KK140798	20	5
27 Jul 1998	KK270798	20	5
	Totals	540	25

Table 2. 16S rDNA Sequencing

A.

KK141097 (14 October 1997)

Colony	Identity	Clinical Significance
1, 3	Uncultured hydrocarbon seep bacterium	Presumably non-pathogenic
2	*Sphingomonas pituitosa*	UTI, septicemia, wound infections (nosocomial)
4,5	*Paracraurococcus ruber*	Non-pathogenic

B.

KK191097 (19 October 1997)

Colony	Identity	Clinical Significance
1, 2	Manganese-oxidizing bacterium	Non-pathogenic in humans
3	Aquatic bacterium	Unknown
4	*Methylobacterium* sp.	*Methylobacterium* species have been reported in septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, and other infections as well as pseudoinfections
5	Alpha proteobacterium	Presumptively non-pathogenic

C.

KK141197 (14 November 1997)

Colony	Identity	Clinical Significance
1, 2	*Drosophila melanogaster*	None (Fruit Fly)
3, 5	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample.
4	Uncultured bacterium	Unknown

D.

KK221197 (22 November 1997)

Colony	Identity	Clinical Significance
1	*Comamonas* sp.	Bacteraemia, conjunctivitis
2	*Alkalispirillum mobilis*	Not known to be pathogenic in humans
3	*Staphylococcus lentus*	Not known to be pathogenic in humans
4	Unidentified eubacterium	Unknown
5	Paracoccus sp.	Not known to be pathogenic in humans

E.

KK201297 (20 December 1997)

Colony	Identity	Clinical Significance
1	Hydrothermal vent eubacterium	Presumably non-pathogenic
2	Dietzia sp.	Non-pathogenic
3	*Ultramicrobacterium* strain	Presumably non-pathogenic
4	Hydrothermal vent eubacterium	Presumably non-pathogenic
5	*Leifsonia poae*	Non-pathogenic

F.

KK040198 (04 January 1998)

Colony	Identity	Clinical Significance
1	*Salinicoccus roseus*	Non-pathogenic in humans
2	*Phenylobacterium immobile*	Not known to be pathogenic in humans
3	Uncultured Antarctic bacterium	Unknown
4	*Lentzea flavoverrucoides*	Non-pathogenic in humans
5	*Lactobacillus* species	bacteraemia, endocarditis, UTI

G.

KK230198 (23 January 1998)

Colony	Identity	Clinical Significance
1	*Bradyrhizobium* genospecies	Non-pathogenic
2	*Thauera selenatis*	Not known to be pathogenic in humans
3	Uncultured bacterium	Unknown
4	*Janthinobacterium agaricidamnosum*	Not known to be pathogenic in humans
5	*Polyangium vitellinum*	Non-pathogenic

H.

KK270198 (27 January 1998)

Colony	Identity	Clinical Significance
1	*Taxeobacter ocellatus*	Non pathogenic
2	*Methylobacterium* sp.	*Methylobacterium* sp. have been reported in septicemia, continuous ambulatory peritoneal dialysis-related peritonitis , other infections, and pseudoinfections
3	Uncultured bacterium	Unknown
4	*Lactobacillus reuteri*	bacteraemia, endocarditis, UTI
5	*Craurococcus roseus*	Non-pathogenic

I.

KK040298 (04 February 1998)

Colony	Identity	Clinical Significance
1	Shuttle vector	Laboratory error
2	Expression vector pRIBOTEX	Laboratory error
3	Unknown bacterium	Unknown
4	Synthetic transposon	Non-pathogenic
5	*Cyanothece*	Green algae

J.

KK130298 (13 February 1998)

Colony	Identity	Clinical Significance
1	*Juniperus virginiana* chloroplast	None, plant chloroplast
2	*Streptosporangium* sp.	Non-pathogenic
3	Bacterial species	Unknown
4	Rhizobium sp.	Non-pathogenic
5	*Methylobacterium* sp.	*Methylobacterium* sp. have been reported in septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, other infections, as well as, pseudoinfections

K.

KK180298 (18 February 1998)

Colony	Identity	Clinical Significance
1	Cloning vector	Laboratory error
2	*Tessaracoccus bendigoensis*	Not known to be pathogenic in humans
3	*Methylobacterium* sp.	*Methylobacterium* sp. have been reported in septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, other infections, as well as, pseudoinfections
4	*Sinorhizobium fredii*	Non-pathogenic
5	*Lactobacillus gasseri*	Non-pathogenic

L.

KK100398 (10 March 1998)

Colony	Identity	Clinical Significance
1	*Juniperus virginiana*	None, plant chloroplast
2	Unidentified eubacterium	Unknown
3	Butyrate-producing bacterium	Not known to be pathogenic in humans
4	*Brachybacterium nesterenkovii*	Non-pathogenic
5	*Paenibacillus polymyxa*	Septicemia, meningitis, pneumonia

M.

KK120398 (12 March 1998)

Colony	Identity	Clinical Significance
1	Unidentified organism	Unknown
2	*Rhizobium* sp.	Non-pathogenic
3	*Clostridium difficile*	Causes diarrhea in humans
4	*Propionibacterium acnes*	Causes acne vulgaris in humans
5	*Acinetobacter* sp.	Septicemia, UTI, wound infections abscesses, endocarditis, meningitis, osteomyelitis

N.

KK170398 (17 March 1998)

Colony	Identity	Clinical Significance
1	*Chlorella saccharophila*	Non-pathogenic
2	uncultured eubacterium	Unknown
3	*Xanthomonas* sp.	Bacteraemia
4	*Eubacterium halii*	wound infection, abscesses, septicemia, periodontitis
5	*Geodermatophilus obscurus*	Not known to be pathogenic in humans

O.

KK210498 (21 April 1998)

Colony	Identity	Clinical Significance
1, 2	*Nicotiana plumbaginifolia* chloroplast	None, plant chloroplast
3	unidentified bacterium	Unknown
4, 5	*Juniperus virginiana* chloroplast	None, plant chloroplast
	Alnus incana	None, plant (grey alder)
	Globicatella sp.	UTI, bacteraemia
	Paracoccus marcusii	Non pathogenic
	Bacillus subtilis	Has been implicated in food-borne illnesses
	Pinus thunbergii chloroplast	None, plant chloroplast

P.

KK230498 (23 April 19980)

Colony	Identity	Clinical Significance
1	*Staphylococcus lentus*	Not known to be pathogenic in humans
2	*Zea mays*	None: corn
3	*Methylobacterium* sp.	*Methylobacterium* sp. have been reported out to septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, other infections, as well as, pseudoinfections.
4	*Nicotiana plumbaginifolia* chloroplast	None, plant chloroplast
5	*Streptococcus salivarius*	Implicated in infections in neuropenic patients

Q.

KK300498 (30 April 1998)

Colony	Identity	Clinical Significance
1, 7	*Nicotiana plumbaginifolia* chloroplast	None, plant chloroplast
2, 4, 5	*Pinus thunbergii* chloroplast	None, plant chloroplast
3	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
6, 8, 10	*Methylobacterium extorquens*	*Methylobacterium* sp. have been reported out to septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, other infections, as well as, pseudoinfections
9	*Yersinia pestis*	agent of plague

R.

KK120598 (12 May 1998)

Colony	Identity	Clinical Significance
1, 2, 4, 5, 8	*Bacillus subtilis*	Has been implicated in food-borne illnesses
3	*Francisella tularensis*	agent of tularemia
6	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although B. anthracis was not detected in the sample
7	*Bacillus badius*	Non-pathogenic
9	*Flavobacterium balustinum*	Not known to be a human pathogen
10	*Acidovorax konjaci*	Wound infection, UTI, bacteraemia, meningitis, septic arthritis

S.

KK140598 (14 May 1998)

Colony	Identity	Clinical Significance
1	*Caulobacter subvibrioides*	Non-pathogenic
2	*Secale cereale* mitochondrion	None: plant (rye)
3	Uncultured soil bacterium	Unknown
4	*Pinus thunbergii* chloroplast	None, plant chloroplast
5	*Pantoea cedenensis*	Not know to cause disease in humans

T.

KK170598 (17 May 1998)

Colony	Identity	Clinical Significance
1	*Spiroplasma* sp.	None, insect and plant pathogen
2	Plastid transformation vector	Laboratory error
3, 4	Ixodes scapularis endosymbiont	Deer tick (may carry Lyme Disease)
5	CDC Group DF-3 16S	Abscesses, bite infections

U.   KK310598 (31 May 1998)

Colony	Identity	Clinical Significance
1	Rhizobium genospecies	Non-pathogenic
2	*Acidovorax avenae* subspecies	Wound infection, UTI, bacteraemia, meningitis, septic arthritis
3	*Taxeobacter gelupurpurascens*	Non-pathogenic
4	*Janthinobacterium lividum*	Non-pathogenic
5	*Craurococcus roseus*	Non-pathogenic

V.   KK180698 (18 June 1998)

Colony	Identity	Clinical Significance
1,3,4,5,6,8,10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
2, 7, 9	*Bacillus subtilis*	Has been implicated in food-borne illnesses

W.   KK300698 (30 June 1998)

Colony	Identity	Clinical Significance
1,2,4,6,10	*Bacillus subtilis*	Has been implicated in food-borne illnesses
3,8,9	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
5	*Brevibacillus choshinensis*	Food poisoning, bacteremia
7	Unidentified eubacterium	Unknown

X.   KK060798 (06 July 1998)

Colony	Identity	Clinical Significance
1	*Bacillus subtilis*	Has been implicated in food-borne illnesses
2, 3, 8	*Renibacterium salmoninarum*	Causes kidney disease in salmon
4, 6, 10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
5	*Kurthia zopfii*	endocarditis, bacteraemia
7	*Paenibacillus amylolyticus*	Septicemia, meningitis, pneumonia
9	*Bacillus pseudofirmus*	Non-pathogenic

Y.

KK140798 (14 July 1998)

Colony	Identity	Clinical Significance
1, 6, 7, 8	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
2	*Brevibacillus choshinensis*	Food poisoning, bacteremia
3, 5	*Clostridium subterminale*	*Clostridium* species are commonly encountered in infections of the abdomen
4	*Paenibacillus amylolyticus*	Septicemia, meningitis, pneumonia,
9	*Renibacterium salmoninarum*	Causes kidney disease in salmon
10	*Bacillus sphaericus*	Non-pathogenic

Z.

KK270798 (27 July 1998)

Colony	Identity	Clinical Significance
1,3,6,10	*Paracoccus aminophilus*	Non-pathogenic
2	*Renibacterium salmoninarum*	Causes kidney disease in salmon
4,5,8	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
7, 9	*Bacillus subtilis*	Has been implicated in food-borne illnesses

Appendix C

KUWAIT

Air samples were collected in Kuwait by either IBADS or the Portal Shield from February 1998 to April 1999. From Kuwait a total of 519 samples were received. This represents eighteen separate sampling days over a fourteen-month period (collections on the IBADs were approximately every hour for 20 hours per day). The sample dates, sample identifier and number of samples collected on each date are listed in Table 1.

RESULTS

Hourly samples collected on a specific day were pooled to give a daily sample. After pooling, daily samples were subjected to PCR amplification of 16S rDNA. In some cases, that day's sample was also subjected to PCR amplification for specific detection of *B. anthracis, F. tularemia and/or Y. pestis*, as described in section 2.3.

Table 2 (panels A – hh) shows the results of PCR amplification and subsequent sequence comparison of amplicon with Genebank database for best fit in order to determine the bacterial origin of the sequenced gene. Each number, as in Table 1, corresponds to the collection location (KW: Kuwait) and the date of collection (i.e., 060298 was collected 6 February 1998). Shown are the best fit for each of 10 colonies picked for subsequent sequencing. The column, "Identity", is the organism with the highest score in the database comparison (best sequence match). The column labeled, "Clinical significance", gives a brief descriptive comment of identified microorganism.

In addition to amplification and sequencing of the bacterial 16S rDNA for bacterial identification, samples from each day were subjected to analysis by PCR using agent specific primers. PCR assays specific for *B. anthracis, Francisella tularensis and Yersinia pestis* were conducted, as described previously. From Kuwait samples, neither *B.* anthracis, *Y. pestis* or *F. tularemia* were detected either by specific PCR. However, *F. tularensis* was found in a sequencing sample from 15 December, 1998. The fact it was identified by sequencing and not by PCR is not too surprising. When an organism is present at very low levels, it sometimes takes several PCR reactions to get a positive reaction. If the organism was present at a sufficiently low concentration in the environment, it is not unlikely that it was not identified in the specific PCR. However, at that low level, the fact it was chosen and sequenced is by pure chance.

Detected Microflora

Examination of the organisms identified reveals only one organism that would be considered a biological agent: *F. tularensis* identified in the 15 December sample. As discussed above, it is unlikely this is evidence of a biological attack, but the detection level of these systems are low enough to detect organisms which are present in the environment. Inspection of the "Clinical Significance" column of Table 2 suggests many

of the organisms found are pathogens. This is not the case. With the exception of the *F. tularensis*, most of these organisms are normal soil inhabitants, and do no normally cause infection. Organisms such as *Sphingomonas, Stenotrophomonas, Ralstonia, Ochrobactrum, Sphingobacterium, Methylobacterium, Eubacterium, Acidovorax, Burkholderia, Delftia, Comamonas, Afipia, Alcaligenes, Facklamia, Pseudomonas, Pantoea,* and *Peptostreptococcus* all occur normally in the environment. It is only when they are able to penetrate the skin, normally by trauma, that they are capable of infecting humans. Normal sanitary measures such as hand washing, and routine wound cleaning are normally enough to avoid infections with these microorganisms.

A number of bacterial agents have been implicated to be either in development as biological warfare agents by rogue states or have been developed (13). Of primary importance is the use of *B. anthracis*, the causative agent of anthrax due to its lethality and viability even under harsh environmental conditions. Because of the significant threat of use of this agent a great deal of focus, internationally, has been placed on detection of this agent.

The genus *Bacillus* includes many different species of bacteria, although they all share common morphological and physiological traits. However, all members of this genus are Gram-positive rods found primarily as soil saprophytes (many species are found world-wide). As a group, they all form endospores in the presence of specific environmental conditions and are thus likely to be represented in high numbers in environmental samples, worldwide. *Bacillus* spp. have a relatively thick cell wall and are resist to physical disruption, such as osmotic shock. Therefore, members of this genera are very hardy and are able to survive deleterious conditions (i.e., droughts, nutrient limitations, osmotic shock, etc).

Within the *Bacillus* genus, several distinct groups exist based on relatedness of 16S rRNA sequences. In fact, the rRNA sequence of *Bacillus cereus* varies from that of *Bacillus anthracis* by only one base over a total of 1544 bases. Therefore, it is sometimes difficult to actually identify to the species level in the genus *Bacillus* based only on 16S rRNA sequence. Nevertheless, the computer software used to compare sequence data (BLAST) often gives suggested species based on "best fit." However, since virulence of *B. anthracis* resides in the expression of specific gene products (i.e., lethal factor, edema factor, protective antigen, as well as capsular genes, encoded on two relatively small plasmids) confirmation of identity of *B. anthracis* is easily accomplished by analysis for the presence of these genes or gene products (14). Because of the importance of this organism, throughout our sampling events, in addition to 16S rRNA evaluation, specific PCR analysis was carried out in order to confirm the presence *Bacillus anthracis* virulence genes. Also, specific PCR analysis was done to screen for *Y. pestis* using primers specific for the plasminogen activator gene and for *F. tularemia* sung TUL 4 gene primers. In the Kuwait samples, neither *B.* anthracis, *Y. pestis* or *F. tularemia* were detected by either 16S rRNA sequencing or specific PCR.

The results of all samples analyzed are given in Table 2. Presented in Table 2 is the best match from the Genebank comparison. In some cases, sequences of the clone did

not result in an identity of the bacterial origin. This is not surprising since the database used, although constantly being improved, is still not complete for all potential bacterial genera and species. However, other clones did result in scores yielding a bacterial match. For those bacteria that were identified, relatively few were validated threat agents. The low numbers of recognized pathogenic agents is possibly due to low levels of aerosolization of bacteria when the samples were collected.

Table 1. Air samples collected in Kuwait for 20 consecutive hours

Sample Date	Sample Identifier	IBADS	Portal Shield
6 Feb 1998	KW060298	18	
9 Mar 1998	KW090398	18	32
17 Jul, 1998	KW170798	21	38
5 Aug, 1998	KW050898	21	22
19 Aug, 1998	KW190898	21	7
6 Sept, 1998	KW060998	21	6
13 Sept, 1998	KW130998	21	3
25 Oct, 1998	KW251098	22	23
3 Nov, 1998	KW031198	21	
1 Dec, 1998	KW011298	21	8
15 Dec, 1998	KW151298	21	
28 Dec, 1998	KW281298	21	
16 Jan, 1999	KW160199	21	
29 Jan, 1999	KW290199	21	
10 Feb, 1999	KW100299	21	
27 Feb, 1999	KW270299	21	
15 Mar, 1999	KW150399	21	
7 Apr, 1999	KW070499		28
	Totals	352	167

Table 2. 16S rDNA Sequencing

A.   KW060298 (06 February 1998)

Colony	Identity	Clinical Significance
1	*Stenotrophomonas maltophilia*	Associated with various nosocomial infections (bacteremia, meningitis, wound infection, UTI, etc.)
2, 5	*Bradyrhizobium* sp.	Non-pathogenic
3	MTBE-degrading bacterium	Presumably non-pathogenic
4, 8	*Sphingomonas paucimobilis*	UTI, septicemia, wound infections (nosocomial)
6	*Sphingomonas aromaticivorans*	UTI, septicemia, wound infections (nosocomial)
7	*Methylobacterium* sp.	Bacteremia, peritonitis
9	*Bradyrhizobium elkanii*	Non-pathogenic
10	*Flavobacterium ferrugineum*	Presumably non-pathogenic

B. KW090398 (09 March 1998)

Colony	Identity	Clinical Significance
1, 4	*Methylobacterium* sp.	Bacteremia, peritonitis
2	*Peptostreptococcus hareii*	Abcesses
3	*Bacillus pseudofirmus*	Non-pathogenic
5	*Eubacterium K*	Wound infection, abscesses, septicemia, periodontitis
6, 7, 9	*Bacillus cohnii*	Non-pathogenic
8	*Acidovorax temperans*	Wound infection, UTI, bacteremia, meningitis
10	*Aminobacter aminovorans*	Presumably non-pathogenic

C. KW170798 (17 July 1998)

Colony	Identity	Clinical Significance
1	*Clostridium lituseburense*	Presumably non-pathogenic
2	*Renibacterium salmoninarum*	Causes kidney disease in salmon
3	uncultured hydrocarbon seep bacterium	Unknown
4	uncultured Antarctic bacterium	Unknown
5	*Kocuria erythromyxa*	From skin flora (doubtful clinical significance)
6	*Actinomyces* sp.	actinomycosis
7	*Streptococcus parasanguis*	endocarditis, bacteraemia, wound infection
8	*Paracoccus denitrificans*	Non-pathogenic
9	*Delftia acidovorans*	Reported cases of bacteremia and endocarditis
10	Uncultured soil bacterium	Unknown

D. KW050898 (05 August 1998)

Colony	Identity	Clinical Significance
1	*Clostridium lituseburense*	Presumably non-pathogenic
2	*Renibacterium salmoninarum*	Causes kidney disease in salmon
3	Unclassified bacterial species	Unknown
4	*Paracoccus kocurii*	Non-pathogenic
5	*Kocuria erythromyxa*	From skin flora (doubtful clinical significance)
6	*Actinomyces* sp.	Actinomycosis
7	unidentified rumen bacterium	Unknown
8	*Paracoccus aminovorans*	Non-pathogenic
9	*Comamonas* sp.	Bacteremia, conjunctivitis
10	Uncultured soil bacterium	Unknown

E.

KW190898 (19 August 1998)

Colony	Identity	Clinical Significance
1, 10	Unidentified bacterium	Unknown
2, 3, 8	*Ralstonia eutropha*	bacteremia, UTI, meningitis, wound infection, peritonitis
4, 5	*Ralstonia* sp.	bacteraemia, meningitis, UTI, wound infection, peritonitis
6, 7, 9	*Burkholderia multivorans*	Lung infection, bacteremia, endocarditis, septic arthritis, UTI (clinical significance of the various taxa in cystic fibrosis patients is unclear)

F.

KW060998 (06 September 1998)

Colony	Identity	Clinical Significance
1	*Alcaligenes latus*	Pneumonia, otitis, UTI, osteomyelitis, bacteraemia
2	*Taxeobacter ocellatus*	Non-pathogenic
3	*Frankia* sp.	Non-pathogenic
4	*Rhizobium* genosp.	Non-pathogenic
5, 9	*Ralstonia eutropha*	Bacteremia, meningitis, UTI, wound infection, peritonitis
6	*Methylobacterium* sp.	Bacteremia, CAPD peritonitis
7	*Flavobacterium ferrugineum*	Presumably non pathogenic
8	*Actinomyces* sp.	Actinomycosis
10	*Burkholderia* sp.	Lung infection, bacteremia, endocarditis, septic arthritis, UTI (clinical significance of the various taxa in cystic fibrosis patients is unclear)

G.

KW130998 (13 September 1998)

Colony	Identity	Clinical Significance
1	*Streptococcus gordonii*	Endocarditis, bacteremia, wound infection
2	*Ralstonia eutropha*	Bacteremia, meningitis, UTI, wound infection, peritonitis
3	Coryneform bacterium	Isolated from clinical material; rarely associated with infections such as septicemia, peritonitis, eye infection, wound infection
4	*Methylobacterium extorquens*	Bacteremia, CAPD peritonitis
5	*Bacillus subtilis*	Has been implicated in food-borne illnesses
6	Unidentified rumen bacterium	Unknown
7	Uncultured High G+C Gram-positive bacterium	Unknown
8	*Streptococcus oralis*	Endocarditis, bacteraemia, wound infection
9	*Bacillus badius*	Non-pathogenic
10	Unidentified rumen bacterium	Unknown

H.

KW251098 (25 October 1998)

Colony	Identity	Clinical Significance
1, 10	Unidentified rumen bacterium	Unknown
2	*Agrobacterium* sp.	Presumably non-pathogenic
3,5,6,8,9	*Burkholderia multivorans*	Lung infection, bacteremia, endocarditis, septic arthritis, UTI (clinical significance of the various taxa in cystic fibrosis patients is unclear)
4, 7	*Ralstonia eutropha*	Bacteremia, meningitis, UTI, wound infection, peritonitis

I.

KW031198 (03 November 1998)

Colony	Identity	Clinical Significance
1, 9, 10	Denitrifying Fe-oxidizing bacteria	Presumably non-pathogenic
2	*Burkholderia multivorans*	Lung infection, bacteremia, endocarditis, septic arthritis, UTI (clinical significance of the various taxa in cystic fibrosis patients is unclear)
3	*Ralstonia eutropha*	Bacteremia, meningitis, UTI, wound infection, peritonitis
4, 5, 7	*Flavobacterium* sp.	Septic arthritis, bone marrow infection
6	*Clostridium propionicum*	Presumably non-pathogenic
8	*Taxeobacter gelupurpurascens*	Non-pathogenic

J.

KW011298 (01 December 1998)

Colony	Identity	Clinical Significance
1	Unknown *Actinomycete*	Actinomycosis
2, 3	Denitrifying Fe-oxidizing bacteria	Presumably non-pathogenic
4	*Aquaspirillum metamorphum*	Non-pathogenic
5, 6, 7	Aquatic bacterium	Presumably non-pathogenic
9	*Intrasporangium calvum*	Non-pathogenic
10	MTBE-degrading bacterium	Non-pathogenic

K.

KW151298 (15 December 1998)

Colony	Identity	Clinical Significance
1	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
2, 6, 7, 8	Unidentified bacterium	Unknown
3	*Azospirillum amazonense*	Non-pathogenic
4	*Sphingomonas adhaesiva*	UTI, septicemia, wound infections (nosocomial)
5	*Francisella tularensis*	Causative agent of tularensis
8	*Afipia broomeae*	septic arthritis, bone marrow infection
10	*Bradyrhizobium* sp.	Non-pathogenic

L.

KW281298 (28 December 1998)

Colony	Identity	Clinical Significance
1, 9	*Stenotrophomonas maltophilia*	Associated with various nosocomial infections- (bacteremia, meningitis, wound infection, UTI and pneumonia
2, 8	*Burkholderia multivorans*	Lung infection, bacteremia, endocarditis, septic arthritis, UTI (clinical significance of the various taxa in cystic fibrosis patients is unclear)
3	*Pantoea endophytica*	UTI, bacteremia, abscess
4	*Aquaspirillum metamorphum*	Non-pathogenic
5	*Rhizobium* genospecies	Non-pathogenic
6	*Sphingomonas* sp.	UTI, septicemia, wound infections (nosocomial)
7	No match	Unknown
10	*Acidovorax* species	Wound infection, UTI, bacteremia, meningitis, septic arthritis

M.

KW160199 (16 January 1999)

Colony	Identity	Clinical Significance
1	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
2, 5, 7, 9	*Sphingomonas* sp.	UTI, septicemia, wound infections (nosocomial)
3, 4	*Ochrobactrum anthropi*	Bcteremia, liver abscess, endophthalmitis
6	*Ochrobactrum intermedium*	Bcteremia, liver abscess, endophthalmitis
8	*Azospirillum amazonense*	Non-athogenic
10	*Stenotrophomonas maltophilia*	Asociated with various nosocomial infections- (acteremia, meningitis, wound infection, UTI and pneumonia)

N.   KW290199 (29 January 1999)

Colony	Identity	Clinical Significance
1	*Sphingobacterium thalpophilum*	Bcteremia, UTI, peritonitis
2, 8	*Afipia broomeae*	Septic arthritis, bone marrow infection
3	*Flavobacterium okeanokoites*	Non-pathogenic
4	*Stenotrophomonas maltophilia*	Associated with various infections (bacteremia, meningitis, UTI, wound infection, and pneumonia)
5	*Caulobacter* sp.	Non-pathogenic
6	*Burkholderia multivorans*	Associated infections (lung, bacteremia, UTI, septic arthritis, endocarditis; clinical significance of the various taxa in cystic fibrosis patients is unclear)
7	*Aerococcus* sp.	Non-pathogenic
9	*Sphingomonas* sp.	UTI, septicemia, wound infections (nosocomial)
10	*Facklamia* sp.	UTI, bacteremia, abscess

O.   KW100299 (10 February 1999)

Colony	Identity	Clinical Significance
1	*Ralstonia* sp.	bacteremia, meningitis, UTI, wound infection, peritonitis
2	*Ralstonia* eutropha	Bacteremia, memingitis, UTI, wound infection, peritonitis
3, 8	*Sphingobacterium thalpophilum*	Bacteremia, UTI, peritonitis
4	*Pseudomonas putida*	Bacteremia, UTI, wound infection, abscesses, septic arthritis, conjunctivitis, endocarditis, meningitis, CAPD peritonitis
5	*Rubrivivax gelatinosus*	Non-pathogenic
6, 10	Denitrifying Fe-oxidizing bacteria	Presumably non-pathogenic
7	*Stenotrophomonas maltophilia*	Associated with various infections (bacteremia, meningitis, UTI, wound infection, and pneumonia)
9	*Sphingomonas* sp.	UTI, septicemia, wound infections (nosocomial)

P.

KW270299 (27 February 1999)

Colony	Identity	Clinical Significance
1	*Rhizobium* genospecies	Non-pathogenic
2	*Afipia broomeae*	Septic arthritis, bone marrow infection
3	Unidentified bacterium	Unknown
4	Denitrifying Fe-oxidizing bacteria	Presumably non-pathogenic
5	*Afipia* genospecies	Septic arthritis, bone marrow infection
6	*Legionella*-like amoebal pathogen	Unknown
7	Unidentified bacterium	Unknown
8, 9	*Sphingomonas* sp.	UTI, septicemia, wound infections (nosocomial)
10	*Leptothrix* sp.	Non-pathogenic

Q.

KW150399 (15 March, 1999)

Colony	Identity	Clinical Significance
1, 4	Denitrifying Fe-oxidizing bacteria	Non-pathogenic
2	*Azospirillum doebereinerae*	Non-pathogenic
3	MTBE-degrading bacterium	Presumably non-pathogenic
5	*Erythromicrobium* sp.	Non-pathogenic
6	*Stenotrophomonas maltophilia*	Associated with bacteremia, UTI, meningitis, wound infection, and pneumonia
7	*Flavobacterium balustinum*	Presumably non-pathogenic
8	*Rhodoplanes* sp.	Non-pathogenic
9	*Sandaracinobacter sibiricus*	Non-pathogenic
10	*Caulobacter* sp.	Non-pathogenic

R.

KW070499 (07 April 1999)

Colony	Identity	Clinical Significance
1	Alpha proteobacterium	Presumably non-pathogenic
2	*Sphingomonas subterranea*	UTI, septicemia, wound infections (nosocomial)
3	*Leishmania major*	Leishmaniasis
4	*Alcaligenes latus*	Pneumonia, otitis, UTI, osteomyelitis, bacteremia
5	Alpha proteobacterium	Presumably non-pathogenic
6, 7, 8	*Ochrobactrum intermedium*	Bacteremia, liver abscess, endophthalmitis
9	*Aquabacterium* sp.	Presumably non-pathogenic
10	Iron-oxidizing lithotroph	Presumably non-pathogenic

Appendix D

Camp Doha, Bahrain

Air samples were collected in Bahrain by either IBADS or the Portal Shield system, discontinuously from February 1998 to April 1999. From Bahrain, we received a total of 516 samples. This represents twenty-five separate sampling days over a fourteen month period (collections on the IBADs were approximately every hour for 18-21 hours per day). The sample dates, sample identifier and number of samples collected on each date are listed in Table 1.

RESULTS

Samples collected on a specific day were pooled to give a daily sample. After pooling, daily samples were subjected to PCR amplification of 16S rDNA. For each collection date the pooled samples were also subjected to PCR amplification for specific detection of *B. anthracis, F. tularemia* and/or *Y. pestis*, as described in section 2.3.

Table 2 (panels A – Y) shows the results of PCR amplification and subsequent sequence comparison of amplicon with Genebank database for best fit in order to determine the bacterial origin of the sequenced gene. Each number, as in Table 1, corresponds to the collection location (BH: Bahrain) and the date of collection (i.e., 260498 was collected 26 April 1998). Shown are the best fit for each of 5 - 10 colonies picked for subsequent sequencing. The column, "Identity", is the organism with the highest score in the database comparison (best sequence match). The column labeled, "Clinical significance", gives a brief descriptive comment of identified microorganism.

In addition to amplification and sequencing of the bacterial 16S rDNA for bacterial identification, samples from each day were subjected to analysis by PCR using agent specific primers. PCR assays specific for *B. anthracis, F. tularensis and Y. pestis* were conducted, as described previously. While *B. anthracis* was never detected, samples collected on 27 February, 1999 were positive for *F. tularensis* and *Y. pestis*. We do not believe this was indicative of a biological attack, rather it demonstrated that the organisms are present in some low background numbers. It is interesting to note that the 16S sequencing from that date also identified *Y. pestis* and *F. tularensis*.

Detected Microflora

A number of bacterial agents have been implicated to be either in development as biological warfare agents by rogue states or have been developed (13). Of primary importance is the use of *B. anthracis*, the causative agent of anthrax due to its lethality and viability even under harsh environmental conditions. Because of the significant threat of use of this agent a great deal of focus, internationally, has been placed on detection of this agent.

The genus *Bacillus* includes many different species of bacteria, although they all share common morphological and physiological traits. Members of this genus are Gram-positive rods found primarily as soil saprophytes (many species are found World-wide). As a group, they all form endospores in the presence of specific environmental conditions and are thus likely to be represented in high numbers in environmental samples, Worldwide. *Bacillus* spp. have a

relatively thick cell wall and are resistant to physical disruption, such as osmotic shock. Therefore, members of this genera are very hardy and are able to survive deleterious conditions (i.e., droughts, nutrient limitations, osmotic shock, etc).

Within the *Bacillus* genus, several distinct groups exist based on relatedness of 16S rRNA sequences. In fact, the rRNA sequence of *B. cereus* varies from that of *B. anthracis* by only one base over a total of 1544 bases. Therefore, it is sometimes difficult to actually identify to the species level in the genus *Bacillus* based only on 16S rRNA sequence. Nevertheless, computer software used to compare sequence data (BLAST) often gives suggested species based on "best fit." Since virulence of *B. anthracis* resides in the expression of specific gene products (i.e., lethal factor, edema factor, protective antigen, as well as capsular genes, encoded on two relatively small plasmids) confirmation of identity of *B. anthracis* is easily accomplished by analysis for the presence of these genes or gene products (14). Because of the importance of this organism, throughout our sampling events, in addition to 16S rRNA evaluation, specific PCR analysis was carried out in order to confirm the presence *B. anthracis* virulence genes. In addition, specific PCR analysis was done to screen for *Y. pestis* using primers specific for the plasminogen activator gene and for *F. tularemia* sung TUL 4 gene primers. In the Bahrain samples, *B.* anthracis was not detected. However, on 27 February 1999 (Table 2, entry U) both *Y. pestis* or *F. tularemia* were detected by both 16S rRNA sequencing and specific PCR. As mentioned earlier, we do not believe this was a deliberate release or attack on US forces in Bahrain. Rather, the sensitivity of the system was able to detect the natural occurrence of these microorganisms.

The results of all samples analyzed are given in Table 2. Presented in Table 2 is the best match from the Genebank comparison. In some cases, sequences of the clone did not result in an identity of the bacterial origin. This is not surprising since the database used, although constantly being improved, is still not complete for all potential bacterial genera and species. However, other clones did result in scores yielding a bacterial match. For those bacteria that were identified, relatively few were validated threat agents. The low numbers of recognized pathogenic agents is possibly due to low levels of aerosolization of bacteria when the samples were collected. Interestingly, however, *Clostridium difficile*, the causative agent of antibiotic-associated diarrhea and pseudomembraneous colitis was detected from samples collected on 18 April, 1998. In addition, several other organisms which *may* cause disease were found, such as *Burkholderia, Ralstonia, Sphingomonas, Brevibacillus, Xanthamonas, Acinetobacter,* and *Veillonella*. All these organisms are normal inhabitants of the soil worldwide. While they may cause an infection, a break in the skin barrier is necessary. Normal sanitary measures such as hand washing, and routine wound cleaning are normally enough to avoid infections with these microorganisms.

Detailed Genebank results with statistical scores are available through the Joint Program Office as compact disks. Also, as previously mentioned, meteorological data collected at the time samples were collected is also available through the Joint Program Office.

Table 1 - Air samples collected in Bahrain for 20 consecutive hours

Sample Date	Sample Identifier	IBADS	Portal Shield
7 Feb 1998	BH070298	18	
11 Feb 1998	BH110298	18	
27 Feb 1998	BH270298	18	
1 Mar 1998	BH010398	18	
11 Mar 1998	BH110398	18	
15 Mar 1998	BH150398	18	
23 Mar 1998	BH230398	18	
4 Apr 1998	BH040498	18	
13 Apr 1998	BH130498	18	
18 Apr 1998	BH180498	18	
26 Apr 1998	BH260498	18	
4 May 1998	BH040598	18	
12 May 1998	BH120598	18	
28 May 1998	BH280598	18	
17 Dec 1998	BH171298	21	3
3 Jan 1999	BH030199	21	3
16 Jan 1999	BH160199	21	3
30 Jan 1999	BH300199	21	3
7 Feb 1999	BH070299	21	3
14 Feb 1999	BH140299	21	3
27 Feb 1999	BH270299	21	3
9 Mar 1999	BH090399	21	3
24 Mar 1999	BH240399	21	3
7 Apr 1999	BH070499	21	3
25 Apr 1999	BH250499	21	3
	Totals	483	33

Table 2. 16S rDNA Sequencing from Bahrain samples

A.

BH 070298 (07 February 1998)

Colony	Identity	Clinical Significance
1	Blood disease bacterium strain R223	Disease is banana blood-specific.
2	Uncultured gamma proteobacterium	Unknown
3	Unidentified eubacterium	Unknown
4	*Acinetobacter johnsonii*	Generally considered to be non-pathogenic to healthy individuals, but may cause infections in debilitated individuals
5	*Cytophaga* sp.	Not known to be a human pathogen

B. BH 110298 (11 February 1998)

Colony	Identity	Clinical Significance
1	*Nicotiana plumbaginifolia* chloroplast	None
2,3,5	*Nicotiana tabacum* chloroplast	None
4	*Pinus thunbergii* chloroplast	None

C. BH 270298 (27 February 1998)

Colony	Identity	Clinical Significance
1	*Taxeobacter ocellatus*	Not known to be pathogenic in humans
2	*Sinorhizobium* species	Not known to be pathogenic in humans
3	*Methylobacterium organophilum*	Some *Methylobacterium* sp. have been reported to cause septicemia, skin ulcers, synovitis and other infections
4	*Methylobacterium* sp.	Occurs mostly in vegetation, but may also be found in hospital environment
5	*Variovorax paradoxus*	Not known to be pathogenic in humans

D. BH 010398 (01 March 1998)

Colony	Identity	Clinical Significance
1,3,4	Uncultured bacterium	Unknown
2	*Arthrobacter* species	Not known to be pathogenic in humans
5	*Phaseolus vulgaris*	Not known to be pathogenic in humans

E.   BH 110398 (11 March 1998)

Colony	Identity	Clinical Significance
1	*Methylobacterium* sp.	Occurs mostly in vegetation, but may also be found in hospital environment
2	Uncultured Bacterium	Unknown
3	Uncultured Alpha Proteobacterium	Unknown
4	Unidentified Eubacterium	Unknown

F.   BH 150398 (15 March 1998)

Colony	Identity	Clinical Significance
1	Bacterium strain	Unknown
2	Unknown organism	Unknown
3	*Acidovorax temperans*	Not known to be pathogenic in humans
4,5	*Ralstonia* sp.	Certain members of the genus associated with wound infections, UTI and bacteremia.

G.

BH 230398 (23 March 1998)

Colony	Identity	Clinical Significance
1,2,5	*Phormidium* sp.	Not known to be pathogenic in humans
3	*Bacillus pseudofirmus*	Not known to be pathogenic in humans
4	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample

H.

BH 040498 (04 April 1998)

Colony	Identity	Clinical Significance
1,2	*Paracoccus aminophilus*	Not known to be pathogenic in humans
3	*Azospirillum aminophilus*	Not known to be pathogenic in humans
4	*Bacillus subtilis*	Implicated in food poisoning and other human and animal infections
5	*Alkali spirillum mobils*	Not known to be pathogenic in humans
6	*Pantoea ananas*	Not known to be pathogenic in humans
7	Uncultured High G+C Gram-positive bacterium	Unknown
8	*Burkholderia multivorans*	Occasionally found in wound infections, UTI, bacteremia
9	*Clostridium propionicum*	*Clostridium* sp. are commonly encountered with infections of the abdomen
10	Alpha proteobacterium	Unknown

I.

BH 130498 (13 April 1998)

Colony	Identity	Clinical Significance
1	*Frateuria aurantia*	Not known to be pathogenic in humans
2	*Bacillus methanolicus*	Not known to be pathogenic in humans
3	Aquatic bacterium	Unknown
4	*Mesorhizobium tianshanens*	Not known to be pathogenic in humans
5	*Actinomycetaceae*	Normal flora in mouth, skin; occasionally associated with infections after trauma

J.

BH180498 (18 April 1998)

Colony	Identity	Clinical Significance
1	*Clostridium difficile*	Causes diarrhea in humans
2	Uncultured bacterium	Unknown
3	*Salinicoccus roseus*	Not known to be pathogenic in humans
4	Unidentified rumen bacterium	Unknown

K.

BH260498 (26 April 1998)

Colony	Identity	Clinical Significance
1	Uncultured Bacterium	Unknown
2	Shuttle Vector	Laboratory error
3,4,5	Activation-tagging vector	Laboratory error

L.

BH040598 (04 May 1998)

Colony	Identity	Clinical Significance
1	Denitrifying Fe-oxidizing bacteria	Not known to be pathogenic in humans
2	*Ralstonia* sp.	Certain members of the genus associated with wound infections, UTI and bacteremia.
3	*Nicotiana tabacum*	Not known to be pathogenic in humans

M.

BH120598 (12 May 1998)

Colony	Identity	Clinical Significance
1	*Thioalcalovibrio denitrificans*	Not known to be pathogenic in humans
2	*Propionibacterium acnes*	Causes acne
3	*Salinicoccus roseus*	Not known to be pathogenic in humans

N.

BH280598 (28 May 1998)

Colony	Identity	Clinical Significance
1	Unidentified eubacterium	Unknown
2	*Dermacoccus nishinomiyaensis*	Not known to be pathogenic in humans
3	*Thermomicrobium roseum*	Not known to be pathogenic in humans
4	*Sphingomonas* sp.	Certain members of the genus associated with wound infections, UTI, bacteremia and nosocomial infections
5	Unidentified bacterium	Unknown

O.

BH171298 (17 December 1998)

Colony	Identity	Clinical Significance
1	*Renibacterium salmoninarum*	Causes kidney disease in salmon
2,9	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
3	*Bacillus benzoevorans*	Not known to be pathogenic in humans
4	*Paracoccus aminophilus*	Not known to be pathogenic in humans
5	*Microscilla furvescens*	Not known to be pathogenic in humans
6	*Streptococcus mitis*	Found in mature dental plaque, however presence may be associated with subacute bacterial endocarditis especially in patients with prosthetic valves
7	*Stenotrophomonas maltophilia*	Nosocomial associated infections (bacteremia, meningitis, wound and UTI)
8	*Staphylococcus capitis*	Found on the adult human head, especially scalp and forehead where sebaceous glands are numerous and well-developed
10	*Propionibacterium acnes*	Causes acne

P.

BH030199 (03 January 1999)

Colony	Identity	Clinical Significance
1	*Rhizobium* sp.	Not known to be pathogenic in humans
2	Unidentified bacterium	Presumptively non-pathogenic
3	*Cupriavidus necator*	Non-pathogen in humans
4	*Bacillus subtilis*	Implicated in food poisoning and other human and animal infections
5	*Microcoleus* sp.	Non-pathogenic
6	Alpha proteobacterium	Unknown

Q.

BH160199 (16 January 1999)

Colony	Identity	Clinical Significance
1,2	*Acidovorax temperans*	Implicated in wound infections and UTI
3	*Stenotrophomonas* sp.	Can infect transplant patients
4,5	Alpha proteobacterium	Unknown

R.

BH300199 (30 January 1999)

Colony	Identity	Clinical Significance
1	*Paracoccus* sp.	Presumably non-pathogenic
2	*Propionibacterium acnes*	Causes acne
3	*Leptolyngbya foveolarum*	Non-pathogenic
4	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected
5	*Methylobacterium rhodium*	*Methylobacterium* sp. have been reported in septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, other infections, as well as, pseudoinfections
6	*Renibacterium salmoninarum*	Causes kidney disease in salmon
7	Unidentified rumen bacterium	Presumably non-pathogenic
8	*Chlamydomonas reinhardtii*	None (green algae)
9	*Facklamia* sp.	Certain members of the genus associated with wound abcesses, UTI, bacteraemia.

S.

BH070299 (07 February 1999)

Colony	Identity	Clinical Significance
1	*Rhodospirilum* sp.	Not known to be pathogenic in humans
2	*Bacillus pseudomegaterium*	Non pathogenic
3,4	*Timone* isolate	Unknown
5	*Rhodospirilum* sp.	Not known to be pathogenic in humans
6	*Solanum nigrum* chloroplast	No, plant chloroplast
7	*Chlorella mirabilis*	No, green algae
8,9	*Clostridium irregularis*	*Clostridium* sp. are commonly encountered in infections of the abdomen
10	*Hydrothermal venteubacterium*	Not known to be pathogenic in humans

T.   BH140299 (14 February 1999)

Colony	Identity	Clinical Significance
1	*Porphyrobacter* sp.	Non-pathogenic
2	*Triticum arstivum* chloroplast	Plant chloroplast
3	*Caulobacter* sp.	Non-pathogenic
4, 10	Unidentified bacterium	Unknown
5	Iron-oxidizing lithotroph	Non-pathogenic
6	*Sandaracinobacter sibiricus*	Non-pathogenic
7	*Xanthomonas campestris*	Possibly causes bacteraemia, cabbage pathogen
8	*Comamonas* sp.	Bacteremia, conjunctivitis
9	*Caulobacter* sp.	Not known to be pathogenic in humans
11	*Propionibacterium acnes*	Causes acne
12	*Acinetobacter* sp.	Generally non-pathogenic for healthy individuals, but may cause infections in debilitated individuals
13	Uncultured eubacterium	Presumably non-pathogenic
14	*Taxeobacter* sp.	Not known to be pathogenic in humans
15, 17	Unidentified soil eubacterium	Related to *Clostridium* sp. ;presumptively non-pathogenic
16	Uncultured hydrocarbon seep bacterium	Unknown
18	*Veillonella dispar*	Abcesses, bacteremia
19	*Bacillus cohnii*	Not known to be pathogenic in humans
20	*Janthinobacterium agaricidamnosum*	Not known to be pathogenic in humans

U.   BH270299 (27 February 1999)

Colony	Identity	Clinical Significance
1	*Francisella tularensis*	Causes ulceroglandular problems typically due to tick bites and contact with infected animals
2	*Pasteuria penetrans*	Not known to cause disease in humans
3	*Streptococcus oralis*	Major species causing infections in neutropenci patients (Viridans streptococci)
4	*Bacillus mycoides*	Causing opportunistic infections and food-borne illness
5, 6	*Bacillus* sp.	Not differentiated from other *Bacillus* genera, although *B. anthracis* was not detected in the sample
7	*Yersinia pestis*	Causes plague in humans and animals
8	*Bacillus cereus*	Causes food-borne illness and opportunistic infections
9	Unidentified bacterium	Unknown

V.   BH090399 (09 March 1999)

Colony	Identity	Clinical Significance
1	Unidentified rumen bacterium	Presumptively non-pathogenic
2	*Clostridium magnum*	Commonly encountered in a variety of polymicrobial infections of the abdomen
3	*Brevibacillus choshinensis*	Food poisoning, bacteraemia
4	*Alkalispirillum mobilis*	Non-pathogenic
5	*Clostridium sporosphaeroides*	*Clostridium* sp. are commonly encountered in infections of the abdomen
6	*Flavobacterium ferrugineum*	Not found in human clinical specimens
7	*Dietzia* sp.	Not pathogenic
8	Uncultured eubacterium	Unknown
9	*Flavobacterium ferrugineum*	Not found in human clinical specimens

W.

BH240399 (24 March 1999)

Colony	Identity	Clinical Significance
1,2,4,5,67,9	*Bacillus subtilis*	Implicated in food poisoning and other human and animal infections
3,11	Unidentified bacterium	Unknown
8	Uncultured soil bacterium	Unknown
10	Uncultured bacterium	Unknown
12	Unidentified eubacterium	Unknown

X.

BH070499 (07April 1999)

Colony	Identity	Clinical Significance
1,5,7	*Bacillus subtilis*	Implicated in food poisoning and other human and animal infections
2	*Brevibacillus choshinensis*	Not known to cause infection
3	*Paracoccus* sp.	Non-pathogenic
4	*Lactobacillus delbrueckii*	Non-pathogenic
6	Uncultured marine bacterium	Unknown
8	*Paracraurococcus ruber*	Not known to cause infections in humans

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Y.

BH250499 (24April 1999)

Colony	Identity	Clinical Significance
1	*Renibacterium salmoninarum*	Causes kidney disease in salmon
2,7,10	*Bacillus* sp.	Not differentiated from other *Bacillus* genera
3	*Leptothrix* sp.	Non-pathogenic
4,5,9	*Bacillus subtilis*	Implicated in food poisoning and other human and animal infections
6	Uncultured bacterium	Unknown
8	*Methylobacterium* sp.	Occurs mostly on vegetation, but may also be found in the hospital environment